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THE UNIVERSITY OF ALBERTA

ACCESSORY CELL FUNCTION IN T CELL INDEPENDENT B CELL ACTIVATION AND TOLERANCE

by

ANIMESH AMART SINHA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES

IMMUNOLOGY

EDMONTON, ALBERTA

SPRING 1986

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ISBN 0-315-30202-X

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Accessory Cell Function in T Cell-Independent B Cell Activation and Tolerance submitted by Animesh Amart Sinha in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Medical Sciences (Immunology).

Supervisor

Bhagirath Singh

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External Examiner

Date April 8, 1986

Dedication

To my father Dr. Birendra Kumar Sinha and my mother Asha Sinha for their unfailing encouragement and support throughout my studies.

Abstract

The vertebrate immune system is capable of recognizing and attacking foreign invaders while remaining unresponsive or tolerant to self tissues. Much evidence is accumulating that accessory cells (including macrophages) play a pivotal role in determining the balance between immunity and unresponsiveness. This thesis describes a series of experiments assessing accessory cell function in B lymphocyte activation and tolerance.

T cell independent B cell tolerance to hapten derivatives of carboxymethyl cellulose (CMC) or methyl cellulose (MC) appears to be controlled by Thy-1$^-$, Ly-2$^-$ adherent accessory (A) cells contained in the spleen or peritoneal fluid. Immunocompetence in nonadherent (NA) normal spleen cells could be restored *in vitro* by irradiated A cells from normal mice. However, NA cells reconstituted with irradiated A cells derived from hapten specifically tolerant mice failed to respond to the same hapten, but responded normally to an immunogenic challenge with another unrelated antigen. A cells that had been preincubated at 4° C with hapten derivatized MC also failed to restore immunocompetence. While preincubation of unfractionated spleen cells with the tolerogen under the same conditions resulted in B cell unresponsiveness, such treatment of NA cells failed to render B cells tolerant. Incubation of MC treated spleen cells with indomethacin or interleukin-1 (IL-1) *in vitro* did not restore immunocompetence to the tolerizing hapten. Furthermore, the presence of carrier specific T cell help did not interfere with the induction of tolerance. However, treatment of A cells from tolerant mice with the reducing agent potassium iodide (KI) *in vitro* restored their capacity to render cultures of NA cells immunocompetent to the relevant hapten. Macrophages (MØ) are known to exert cytotoxic effects upon target cells by the release at close range of oxidative agents. We postulate that hapten derivatized CMC and MC, through unique properties of the carrier, bind to MØ rendering them specifically suppressive for hapten binding B cells.

The function of A cells in thymus independent (TI) B cell activation was investigated using inhibitors of antigen processing and IL-1 secretion as well as homogeneous A cell lines with distinct cell surface and functional characteristics. B cell responses to both type 1 and

type 2 TI (TI-1 and TI-2) antigens were found to be strictly A cell dependent. Only A cells capable of IL-1 secretion could restore responsiveness in A cell depleted spleen cells, regardless of histocompatibility, Ia antigen expression, or antigen processing capability. Moreover, recombinant IL-1 completely replaced A cell function in B cell responses to both TI-1 and TI-2 antigens. Thus, in contrast to T cell activation, IL-1 secretion is the only A cell function required in TI B cell activation.

Preface

This dissertation describes a series of studies on the functions of accessory cells in T cell independent B cell activation and tolerance.

Some of the results shown in *Chapters IV* and *VI* have been published in *Cellular Immunology*. The work described in *Chapter V* has been submitted for publication. Some of the studies present in this thesis were done in collaboration with Dr. K.-C. Lee. Flow cytometric studies (*Chapters V* and *VI*) were done in collaboration with Dr. P. Mannoni and Dr. L. Brox.

Acknowledgements

I wish to thank my supervisor Dr. Erwin Diener for his interest, insightful suggestions, and guidance in all stages of my dissertation research. Dr. Diener is not only an ideal research supervisor, but has been helpful in every aspect of my academic career.

I express my appreciation for the contributions of the members of my supervisory committee, Dr. K.-C. Lee, Dr. B.M. Longenecker, and Dr. B. Singh, in completing this research project. I acknowledge the support of Dr. P. Mannoni, Dr. L. Pilarski, and Dr. T. Wegmann. My thanks are also due to Mrs. J. Dillon, professional officer in the Department of Immunology. My fellow graduate students and post-doctoral colleagues, especially Cynthia Guidos, Dr. L. Francescutti, R. Hunziker, Dr. J. Krowka, and Dr. V. LaPosta, deserve my gratitude for providing an atmosphere of comradeship in the Department.

I would like to express my sincere thanks to my mentors Dr. R.P. Beck, Dr. A.M. Edwards, Dr. G. Hopkinson, Dr. T.A. McPherson, Dr. W.M. Rigal, Dr. D.N.P. Singh, Dr. D.L. Tyrrell, and Dr. W.W. Yakimets for their help and support throughout my medical training at the University of Alberta.

During my four years of stay at the Department of Immunology, I have had the privilege of receiving continual help of the technical and secretarial staff: Dr. D. Gong, L. Linklater, J. Ingels, Dr. S. Sawada, R. Vergidis, K. Forbes, G. Hall, C. Miller, L. Nugent, M. Pelletier, and D. Zinyk. I am especially grateful to Dawn Zinyk for her dedicated effort in the final preparation of the manuscript.

Finally, I am indebted to my parents and my younger brothers, Neel and Smit, for their unconditional support. Our family dog, "Zippy", was a special source of joy and reinforcement.

This work was supported by a grant to Dr. E. Diener and the MRC Group on Immunoregulation from the Medical Research Council of Canada. I am pleased to acknowledge the receipt of a Research Fellowship from the Alberta Heritage Foundation for Medical Research, 1982-1986.

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List of Abbreviations

Immunoglobulins and chemical compounds are named in accordance with the WHO and IUPAC nomenclatures, respectively. Other abbreviations include:

A cell	adherent accessory cell
ABC	antigen binding cell
AFC	antibody forming cell
APC	antigen presenting cell
ATS	anti-thymocyte serum
B cell	bone marrow derived lymphocyte
Ba	*Brucella abortus*
BCDF	B cell differentiation factor
BCGF	B cell growth factor
BGG	bovine gamma globulin
BM-MØ	bone marrow-macrophages
BMF	B cell maturation factor
BSA	bovine serum albumin
BSFp1	B cell stimulating factor-provisional 1
C'	complement
C5	fifth complement component
CMC	carboxymethyl cellulose
CNBr	cyanogen bromide
Con A	Concanavalin A
DC	dendritic cells
DNP	dinitrophenyl
EBV	Epstein Barr Virus
FACS	fluorescence activated cell sorter
FcR	Fc receptor

FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLU	fluorescein
GAT	Glu^{60}Ala^{30}Tyr^{10}
H-2	histocompatibility-2 locus
HBSS	Hanks balanced salt solution
HGG	human gamma globulin
Ia	I region associated antigens
id	idiotype
Ig	immunoglobulin
IL-1	interleukin-1
IL-2	interleukin-2
IL-3	interleukin-3
Ir gene	immune response gene
KI	potassium iodide
KLH	keyhole limpet hemocyanin
LPS	lipopolysaccharide
L90	log 90° light scatter
MØ	macrophage(s)
MC	methylcellulose
MDBSS	Mishell Dutton balanced salt solution
2-ME	2-mercaptoethanol
MHC	major histocompatibility complex
mIg	membrane immunoglobulin
MON	monomeric POL
NA	nonadherent
nu/nu	homozygous nude mice
OVA	ovalbumin

PAC	peritoneal accessory cell
PBA	polyclonal B cell activator
PBS	phosphate buffered saline
PMA	phorbol myristate acetate
RES	reticuloendothelial system
SAC	splenic accessory cell
SD	standard deviation
SGG	sheep gamma globulin
SIRS	soluble immune response suppressor
SRBC	sheep red blood cell
T cell	thymus derived lymphocyte
T_h	T helper cell
T_s	T suppressor cell
TD	thymus dependent
TI	thymus independent
TI-1	thymus independent type-1
TI-2	thymus independent type-2
TNBS	trinitrobenzene sulfonic acid
TNP	trinitrophenyl
TRF	T cell replacing factor
UV	ultraviolet
v/v	volume/volume
xid	X-linked immunodeficiency

I. Introduction

The vertebrate immune system is comprised of a number of regulatory and effector cell types which function synergistically to protect the organism from potentially harmful microorganisms and neoplasms. Cell mediated and humoral (antibody mediated) effector mechanisms have evolved to facilitate the destruction of both intracellular and extracellular pathogens. The two classes of lymphocytes which perform these functions (T and B lymphocytes, respectively) bear clonally distributed receptors for antigen which are specific, at the population level, for an almost infinite array of molecular configurations. However, under normal circumstances, immune responses are generated only against antigens which are recognized as foreign, or nonself. Self-nonself discrimination by immunocompetent cells is vital to prevent autoimmune destruction of self organs and tissues. The process by which lymphocytes learn to distinguish self from nonself antigens remains an enigma, despite having been a dominant theme in immunological investigations of the last three decades.

Experimental analysis of mechanisms involved in the establishment and maintenance of self tolerance has largely focussed on interactions between immunocompetent cells and antigens. However, the cooperation of lymphocytes with nonlymphocyte accessory (A) cells is critical to immune induction and may also be important in self tolerance. One type of A cell is the phagocytic macrophage (MØ), discovered over 100 years ago by Metchnikoff (1884). Although initially ascribed only a minor role in adaptive immunity, MØ and other A cells have gained prominence as being central to immune induction.

In the years since the demonstration that both adherent cells (presumably MØ) (Mosier, 1967) and T cells (Claman *et al*, 1966; Miller and Mitchell, 1967) are required to cooperate for efficient antibody formation, most attention has been focussed on the functions of MØ and other adherent A cells in T cell activation (reviewed in Schwartz *et al*, 1978; Unanue, 1984). Evidence that MØ or their secreted products are directly involved in B cell activation also exists, however, their functions have not been well established. Also poorly understood is the potential role of MØ in B cell tolerance; nevertheless, there is a body of evidence suggesting that, at least for some antigens, MØ may control the balance between immune induction and tolerance (reviewed in Mosier, 1976; Pierce, 1980). The objective of this thesis is to investigate the role of MØ and other adherent, nonlymphocyte, A cells in thymus or T cell independent (TI) B cell activation and tolerance. This chapter will begin with a general overview of B cell activation, followed by a review of the literature pertaining to the mechanisms involved in B cell tolerance. Particular emphasis is placed on the role of A cells in TI activation and tolerance.

A. B Cell Activation

Overview

In mammals, bone marrow derived (B) and thymus derived (T) lymphocytes arise from a common progenitor in the bone marrow. Subsequently, they complete the antigen independent phase of their development in the thymus and bone marrow, respectively, prior to migration to the spleen, lymph nodes, and other secondary lymphoid organs. Both T and B lymphocytes bear clonally distributed receptors for antigen, as demonstrated by radioactive antigen suicide of immunocompetent cells (Ada and Byrt, 1969; Basten *et al*, 1971). This finding validated a major tenet of Burnet's Clonal Selection Theory, namely, that lymphocyte clones bear pre-existing receptors of a single antigenic specificity (Burnet, 1957, 1959). Ehrlich (1900) first proposed that cell bound immunoglobulin (Ig) serves as the B cell receptor for antigens, however, this was not formally demonstrated until the 1970's (Raff *et al*, 1970; Warner *et al*, 1970; Feldmann and Diener, 1971).

The genes encoding the light and heavy chains of the Ig receptor are composed of variable, joining and constant region elements which are separate in the germline but rearrange during an early phase of B cell development to form a functional Ig gene (reviewed in Honjo, 1983). Allelic exclusion of the Ig genes on one of the chromosomes ensures that each B cell expresses Ig receptors of only one specificity. The existence of a large repertoire of variable region genes, in addition to combinatorial and junctional diversity and somatic mutation, results in the generation of an extremely diverse repertoire of antigen specificities. Five distinct Ig classes (IgM, IgD, IgG, IgA, IgE) provide for functional diversity of antibody mediated effector mechanisms.

After migration to secondary lymphoid organs, B cells exist in a resting (G_0) state and do not divide. Exit from G_0 begins upon the binding of antigen or anti-Ig to Ig receptors (reviewed in Cambier *et al*, 1985). This results in a complex cascade of events which has been separated into three distinct phases - induction, proliferation or clonal expansion, and maturation of B cells into antibody forming cells (AFC), each being regulated independently (Dutton, 1975a; Schimpl and Wecker, 1975; Williamson, 1975; Melchers and Lernhardt, 1985). Clonal expansion and differentiation are regulated separately by antigen nonspecific T cell and A cell derived soluble factors (Falkoff *et al*, 1982; Jaworski *et al*, 1982; reviewed in Howard *et al*, 1984). A critical regulatory aspect of the activation cascade features the induction of responsiveness to a particular signal only by events in the cascade immediately preceding its point of action (Schimpl and Wecker, 1975; Andersson *et al*, 1980, 1981; Julius *et al*, 1982; Zubler and Glasebrook, 1982, Noelle *et al*, 1983; Swain *et al*, 1983; Kishimoto *et al*, 1984).

Induction

Induction refers to the phenotypic changes which occur in the B cell following the binding of antigen, anti-Ig, or mitogens which are indicative of exit from the G_0 (resting) phase of the cell cycle. Antigen and anti-Ig induced B cell activation require cross-linking of mIg receptors, since monovalent haptens (Monroe and Cambier, 1983a) and F(ab') fragments (Monroe and Cambier, 1983b) which bind B cells, are not stimulatory. Studies with conjugates of various hapten densities (reviewed in Feldmann *et al*, 1975), size-fractionated linear hapten substituted polymers (Dintzis *et al*, 1976, 1982, 1983), and different concentrations of anti-IgM (DeFranco *et al*, 1985), have led to the proposal that a critical threshold of cross-linking is required to deliver a transmembrane activation signal to the B cell. Others have suggested that the mIg receptor merely serves as an antigen focussing device and plays no role in signal transduction (Coutinho and Möller, 1974 ; Coutinho *et al*, 1984). Recent studies have also indicated, however, that the binding of multivalent antigens, anti-Ig, and some mitogens, but not monovalent hapten, induces membrane depolarization, increased I region associated (Ia) antigen expression, calcium ion influxes, phosphatidyl inositol hydrolysis, serine esterase activation, and protien kinase C activation in resting G_0 B cells (Kiefer *et al*, 1980; Pozzan *et al*, 1982; Kishi *et al*, 1983; Monroe and Cambier, 1983a,b,c; Coggeshall and Cambier, 1984; Monroe *et al*, 1984; Bijsterbosch *et al*, 1985; Monroe and Kass, 1985; Ransom and Cambier, 1986; reviewed in Cambier *et al*, 1985). The importance of such events in transmembrane signalling by ligand-receptor interactions on other cell types is well established (reviewed in Cheung, 1980; Berridge and Irvine, 1984). However, the signal transducing function of the mIg receptor remains controversial (Coutinho *et al*, 1984; LoCascio *et al*, 1984; Forni, 1985; Tony *et al*, 1985), primarily because B cell activation can be accomplished by mIg independent mechanisms, such as mitogen activation (reviewed in Andersson *et al*, 1972; Coutinho and Möller, 1975) and direct interactions with T cells (Cammisuli *et al*, 1978; Andersson *et al*, 1980; Augustin and Coutinho, 1980; Schreier *et al*, 1980; Tse *et al*, 1981; Coutinho *et al*, 1984; DeFranco *et al*, 1984; Kishimoto *et al*, 1984; Tony and Parker, 1985).

The activation of these initial B cell events by thymus dependent (TD) antigens alone is not sufficient, however, to drive a resting G_0 B cell into the G_1 phase of the cell cycle; major histocompatibility complex (MHC) restricted, antigen specific T cell help is also required (Cambier *et al*, 1982; Monroe and Cambier, 1983a; Kishimoto *et al*, 1984). This finding elucidates the physiological basis of the need for T-B collaboration (Claman *et al*, 1966; Miller and Mitchell, 1967). This T cell derived "helper" signal can be delivered by a number of different means, including T_h specific for allogeneic Ia (Hamoaka *et al*, 1973; Schrader, 1973b; Julius *et al*, 1982), syngeneic Ia (Clayberger *et al*, 1984; Saito and Rajewsky, 1985), or antigen in association with Ia (LoCascio *et al*, 1984; Lanzavecchia, 1985; Tony and Parker, 1985) on the surface of antigen or anti-Ig activated B cells. Unlike TD antigens, TI antigens (Monroe and Cambier, 1983a) and some mitogens initiate the $G_0 \rightarrow G_1$ transition, obviating the need for antigen specific MHC restricted T cell help. However, such antigens or mitogens are not truly TI, as later stages in the cell cycle require antigen nonspecific T cell factors (Jaworski *et al*, 1982; Zubler and Glasebrook, 1982; Endres *et al*, 1983; Mond *et al*, 1983; Pike and Nossal, 1984). Thus, the B cell cycle features several "restriction points", at which various factors are required to act in order for the cycle to continue (Melchers and Lernhardt, 1985).

Following the binding of multivalent antigens (Diener and Paetkau, 1972) or anti-Ig (Pernis *et al*, 1970; Taylor *et al*, 1971) to B cells, membrane Ig (mIg) receptors become redistributed into small aggregates (patches) which coalesce into one large cap which is subsequently endocytosed or shed, a process known as modulation (reviewed in Unanue and Karnovsky, 1973). Receptor re-expression begins within a few hours under normal circumstances, and is back to normal levels after 24 hours (Unanue *et al*, 1972). Capping is inhibited by Concanavalin A (Con A) (Yahara and Edelman, 1972) or metabolic inhibitors, but not by microtubule disrupting agents, such as colchicine (Unanue and Karnovsky, 1973). Other cytostructural elements, such as microfilaments, are required for capping, cell motility, and receptor endocytosis (Schreiner and Unanue, 1976; Bourguignon and Singer, 1977; Braun and Unanue, 1980). Capping itself, however, is not sufficient for triggering since Con A inhibited B cell capping *in vitro*, but did not prevent antibody secretion (Lee *et al*, 1973). Most likely, capping serves to clear antigen from the cell surface.

Control of Proliferation and Differentiation by Soluble Factors

T cell replacing activity for *in vitro* antibody responses in supernatants of activated T cells was first demonstrated by Dutton (1971) and Schimpl and Wecker (1972). This activity was termed T cell replacing factor (TRF) and was postulated to be a differentiation factor since it acted late in the activation cascade. Subsquently, a number of T cell and A cell derived soluble factors have been implicated in both the proliferative and differentiative phases of the B cell activation cascade (reviewed in Marrack *et al*, 1982; Swain *et al*, 1982; Howard and Paul, 1983; Corbel and Melchers, 1984; Howard *et al*, 1984; Kehrl *et al*, 1984; Kishimoto *et al*, 1984; Kishimoto, 1985). Others, termed B cell maturation (BMF) or γ factors (reviewed in Melchers and Andersson, 1984), appear to polyclonally stimulate resting B cells, in the absence of antigen, to mature into AFC (Jelachich *et al*, 1984; Leclercq *et al*, 1984; 1986; Sidman *et al*, 1984a,b; Thompson *et al*, 1985; Sherris and Sidman, 1986; reviewed in Howard *et al*, 1984), in some cases without proliferation (Melchers *et al*, 1980). Recently, the maturational and proliferative activities of some BMF preparations have been separated (Lernhardt *et al*, 1982; Sidman *et al*, 1984b; Sherris and Sidman, 1986). Thus, BMF can polyclonally stimulate B cells to secrete Ig in the absence of proliferation.

Several distinct factors, generally called B cell growth factors (BCGFs), which function in the proliferative phase of the activation cascade, have been described. B cell stimulating factor-provisional 1 (BSFp1, also called BCGF and BCGF-1) was first described by Howard *et al* (1982) as a factor present in the supernatant of the phorbol myristate acetate (PMA) stimulated EL-4 thymoma which augmented B cell proliferation induced by low concentrations of anti-μ. This factor is distinct from interleukin-1 (IL-1), interleukin-2 (IL-2), and interleukin-3 (IL-3) (Howard and Paul, 1983; Howard *et al*, 1984; Ohara and Paul, 1985) and synergizes with IL-1 in anti-μ induced B cell proliferation (reviewed in Howard and Paul, 1983). Leandersson *et al* (1982) and Lernhardt *et al* (1982) have described similar factors produced by T cell hybridomas which are active in lipopolysaccharide (LPS) induced B cell proliferation. Other sources of this factor include alloreactive T cell clones (Puré *et al*, 1981), long term T cell lines (Howard *et al*, 1983a) and Epstein Barr Virus (EBV) transformed human B cells (Gordon *et al*, 1984). Human BCGFs, active in anti-Ig (Yoshizaki *et al*, 1982; Okada *et al*, 1983) or *Staphylococcus aureus* Cowan strain induced B cell proliferation (Muraguchi and Fauci, 1982) have also been described. Though not proven, these various BCGF activities can most likely be ascribed to a single factor on the basis of similar molecular weights and isolectric points (reviewed in Howard *et al*, 1984). A second type of human and murine BCGF, termed BCGF-II, possessing distict molecular and functional characteristics, has been described (Swain and Dutton, 1982; Swain *et al*, 1983; Okada *et al*, 1983). The two human BCGFs acted synergistically in anti-Ig induced B cell proliferation (Okada *et al*, 1983). The murine factor was active on dextran-sulfate activated, but not anti-Ig activated B cells (Swain *et al*, 1983).

Contrary to initial reports, the activity of BSFp1 is not restricted to the enhancement of proliferation in activated B cells. Oliver *et al* (1985) and Rabin *et al* (1985) reported that

BSFpl treatment of resting B cells in the absence of anti-μ caused an increase in cell volume and rendered B cells more susceptible to anti-μ induced activation. Moreover, BSFpl induced increased expression of Ia antigens on small resting B cells (Noelle *et al*, 1984). Thus, BSFpl may have a broader range of biological activity than initially reported. Other factors previously thought to act only on activated B cells, have also recently been shown to have effects on small G_0 B cells (Leclercq *et al*, 1984, 1986; Ralph *et al*, 1984; Roehm *et al*, 1984; Bich-Thuy and Fauci, 1985). Thus, the notion that B cells express receptors for growth and differentiation factors only after the activation cascade has been initiated will have to be reconsidered.

Recent studies also suggest that T cell growth factor, or IL-2, previously thought to act only on T cells, is important in B cell proliferation (Leibson *et al*, 1981, 1982; Swain *et al*, 1981; Parker, 1982; Kappler *et al*, 1983; Roehm *et al*, 1983; Mingari *et al*, 1984; Zubler *et al*, 1984; Mond *et al*, 1985b). Consistent with these findings, some activated normal (Malek *et al*, 1983; Mingari *et al*, 1984; Tsudo *et al*, 1984; Waldmann *et al*, 1984; Muraguchi *et al*, 1985) or leukemic (Korsmeyer *et al*, 1983; Waldmann *et al*, 1984) B cells display receptors for IL-2. However, the role of IL-2 as a growth factor distinct from other BCGFs remains controversial, since in most cases the supernatants used contained multiple factors and the responding cell populations were not pure. Moreover, some activated B cells did not absorb radiolabeled IL-2 (Leibson *et al*, 1981; Muraguchi and Fauci, 1982; Okada *et al*, 1983) and not all B cell tumors or activated B cells displayed IL-2 receptors (Robb *et al*, 1981; Osawa and Diamantstein, 1984; Ralph *et al*, 1984). Whereas some studies reported that IL-2 receptors on activated B and T cells were similar in number, affinity for IL-2, and molecular weight (Mingari *et al*, 1984; Waldmann *et al*, 1984; Zubler *et al*, 1984; Lowenthal *et al*, 1985; Prakash *et al*, 1985), others demonstrated that IL-2 receptors on B cells were lower in number and affinity for IL-2 (Muraguchi *et al*, 1985). Ralph *et al* (1984) suggested that the IL-2-B cell interaction is mediated *via* a receptor distinct from that for IL-2 on T cells. Finally, IL-2 has been shown to induce BCGF production from T cells (Howard *et al*, 1983a), thus raising the possibility that the observed effects of IL-2 on B cells are mediated indirectly *via* T cells. Evidence for this possibility exists (Miedema *et al*, 1985); however, many recent studies, using recombinant IL-2

(Pike *et al*, 1984; Zubler *et al*, 1984; Bich-Thuy *et al*, 1985; Mingari *et al*, 1985; Nakagawa *et al*, 1985) and B cell cloning (Pike *et al*, 1984) or tumor systems (Nakagawa *et al*, 1985, 1986), have provided substantial evidence that IL-2 acts directly on B cells.

In addition to proliferation cofactors, several differentiation cofactors (TRFs or B cell differentiation factors, BCDFs) have been described which are essential for the induction of Ig secretion by activated B cells (reviewed in Howard and Paul, 1983; Howard *et al*, 1984; Kishimoto *et al*, 1984; Kishimoto, 1985). Jaworski *et al* (1982) found that a T cell derived factor was required to induce LPS activated, proliferating B cells to differentiate into AFC. At least two distinct TRFs, B15-BCDF-nak and EL-BCDF-nak, which appear to act sequentially (Howard and Paul, 1983) are needed to drive anti-μ activated B cells to Ig secretion (Nakanishi *et al*, 1983). Human BCDFs have also been described (reviewed in Kerhl *et al*, 1984; Kishimoto *et al*, 1984; Mayer *et al*, 1984; Kishimoto, 1985) but their relationship with the murine BCDFs has not been established. Another class of BCDFs mediate Ig class switching in B cells (reviewed in Bergstedt-Lindquist *et al*, 1984; Vitetta *et al*, 1984). Lymphokines such as IL-1 (to be discussed separately), IL-2 (Ralph *et al*, 1984; Pike *et al*, 1984; Waldmann *et al*, 1984; Bich-Thuy and Fauci, 1985; Kishimoto, 1985, Muraguchi *et al*, 1985; Nakagawa *et al*, 1985, 1986), and interferons (Harfast *et al*, 1981; Leibson *et al*, 1981, 1982, 1984; Johnson and Torres, 1983; Kappler *et al*, 1983; Rodriguez *et al*, 1983; Roehm *et al*, 1983; Zlotnik *et al*, 1983; Sidman *et al*, 1984a; Brunswick and Lake, 1985; Nakagawa *et al*, 1985, 1986; reviewed in Friedman and Vogel, 1984), which have well established functions in other immune responses, have also been implicated as B cell differentiation factors.

While it is clear from the above discussion that much progress has been made in the identification of soluble factors required for B cell proliferation and differentiation, several problems remain. The assignation of a factor's activity to a particular point in the activation cascade is sometimes difficult since some factors, such as IL-1, IL-2, γ-interferon and BSFp1 have multiple activities. Thus Pike *et al* (1984) and Pike and Nossal (1985a,b) have recently challenged the action that cytokines involved in the B cell activation cascade can be classified as strictly activation, growth, or differentiation factors. Using a cloning system for single hapten specific B cells, they have reported that several alleged growth or differentiation factors caused both growth and differentiation. Demonstrations of synergy between various factors also make it difficult to ascribe a particular activity to a discrete factor. Moreover, it is not clear whether factors from distinct cellular sources and described in slightly different assay systems, yet displaying similar molecular and functional characteristics, represent heterogeneous forms of the same factor or discrete factors with separately identifiable functions. Recently, Ohara *et al* (1985) reported that BSFp1, purified to homogeneity, was active in BCGF-1 and BCDFγ assays, and also induced increased Ia expression on resting B cells (Ohara *et al*, 1985). Subsequently, this factor has been produced using recombinant DNA technology (Noma *et al*, 1986) and shown to possess all three of these activities. Furthermore, certain factors, such as IL-3 and eosinophil differentiation factor, possess biological activity for both lymphoid and myeloid cell types, and thus have been given a multitude of different names, resulting in much confusion (Ihle *et al*, 1983; Palacios *et al*, 1984; Sanderson *et al*, 1986). Through the recent availability of cloned or highly purified growth factors, it is now clear that a single factor can have diverse effects both on a single cell type (depending upon the activation state) as well as on cells of different hemopoietic lineages.

A further source of conflicting experimental data has resulted from the use of different modes of B cell triggering. Thus, in disagreement with other reports (Leandersson *et al*, 1982; Lernhardt *et al*, 1982), Jaworski *et al* (1982) and Inazawa *et al* (1985) could not demonstrate a role for BCGF in LPS induced B cell proliferation. Moreover, a requirement for differentiation factors by antigen affinity purified B cells activated with LPS depended upon which antigen specificity had been selected (Inazawa *et al*, 1985). Similarly, Mond *et al* (1985a) demonstrated that recombinant γ-interferon was either inhibitory or enhancing for B cell activation by soluble *vs* Sepharose bound anti-Ig, respectively. Thus, it is clear from the above discussion that several problems must be resolved before a unifying scheme for cytokine involvement in the B cell activation cascade can be formulated.

MHC Restriction of T_h Cell-B Cell Interactions

Molecules of the major histocompatibility complex (MHC) are encoded by genes within the histocompatibility-2 (H-2) locus on chromosome 17 in the mouse. Phlyogenetic studies have revealed that MHC-like antigens are important for self-nonself discrimination in colonial protochordates (Scofield *et al*, 1982). The requirement for MHC molecules in the cell-cell interactions in immune induction most likely reflects an evolutionary refinement of this primordial function. T lymphocytes recognize foreign antigens only in association with self class I or class II MHC genes, a phenemenon known as MHC restriction. Similarly, the collaboration of T and B lymphocytes leading to antibody production is most efficient when the T and B cells are of the same MHC haplotype (Kindred and Shreffler, 1972; Katz *et al*, 1973; reviewed in Singer and Hodes, 1983). This requirement can be overcome, however, if T and B cells of different genotypes differentiate in an MHC identical environment (von Boehmer *et al*, 1975; Katz *et al*, 1978). Thus, T cells "adaptively differentiate" (Katz, 1980), or learn to recognize a different MHC haplotype, most likely during intrathymic development (reviewed in Bevan and Fink, 1978; Zinkernagel,1978). Adaptive differentiation has also been reported for B cells (Katz *et al*, 1978), but the significance of this is not clear.

Until recently, the physical basis for MHC restriction of T-B interactions was difficult to visualize, given that T_h cells recognize antigen only in the context of class II MHC molecules (reviewed in Schwartz, 1985), and that collaboration is greatly enhanced when the T and B cells are specific for determinants of the same molecule (Rajewsky *et al*, 1969; Raff, 1970; Mitchison, 1971). The latter phenomenon is also known as linked associative recognition, or the hapten-carrier effect. Recent demonstrations that under certain conditions B cells can internalize, process and present antigens to T cells in an MHC restricted manner (functions originally ascribed only to A cells) (Chestnut and Grey, 1981; Chestnut *et al*, 1982; Glimcher *et al*, 1982), and a recent paper by Lanzavecchia (1985), have been illuminating in regard to T-B interactions. A plausible sequence of events is outlined in the following paragraph.

B cells take up antigen through their specific Ig receptors (Chestnut and Grey, 1981; Rock *et al*, 1984; Lanzavecchia, 1985), which begins the activation process (LoCascio *et al*,

1984) and increases B cell Ia expression (Mond *et al*, 1981; Monroe and Cambier, 1983c). In the meantime, processed antigen appears on the B cell surface in association (not necessarily stable) with Ia. Antigen plus Ia specific T helper (T_h) cells are thus focussed onto partially activated B cells specific for determinants on the same antigen, allowing the activation cascade to continue until the next restriction point is reached. B cells which have processed antigens nonspecifically, a much less efficient process (Chestnut and Grey, 1981; Kakiuchi *et al*, 1983; Krieger *et al*, 1985), will activate antigen plus Ia specific T_h, but will not themselves be activated, since the initial antigen-Ig receptor interaction is required to induce responsiveness to T_h signals (Noelle *et al*, 1983; LoCascio *et al*, 1984). This model is consistent with most of the available data and provides a simple, yet elegant, explanation of MHC restricted T-B collaboration and the requirement for hapten-carrier linkage.

B Cell Subpopulations

Numerous studies support the existence of discrete B cell subpopulations, distinguishable on the basis of size (Armstrong and Kraft, 1973; Thompson *et al*, 1984a; Layton *et al*, 1985) or differential responsiveness to mitogens (Gronowicz and Coutinho, 1974; reviewed in McKearn *et al*, 1982) or TD and TI antigens (Playfair and Purves, 1971; Gorczynski and Feldmann, 1975; Jennings and Rittenberg, 1976; Lewis *et al*, 1976; Quintans and Cosenza, 1976; reviewed in Marshall-Clarke and Playfair, 1979). A more recent study, utilizing single, hapten specific B cells, was inconclusive in providing evidence for separate TD and TI precursors due to the strong bystander effects of T cell derived lymphokines on B cells responding to TI antigens (Hebbard *et al*, 1984). It has been suggested that the response of B cells to TD *vs* TI antigens is determined by the presence or absence of mIgD (reviewed in Kettman *et al*, 1979). However, the distinction between TD and TI B cells on the basis of mIgD expression is not tenable, since no difference in responsiveness to TD and TI antigens could be demonstrated in fluorescence activated cell sorter (FACS) purified $mIgD^+$ and $mIgD^-$ subsets (Layton *et al*, 1979a,b).

Spleen cells from mice which have not been deliberately immunized contain small, resting G_0 B cells as well as larger cells which are in various stages of activation (Thompson *et al*, 1984a; Layton *et al*, 1985). The activation requirements of the small and large subsets differ. Small G_0 B cells exhibit a strict requirement for antigen plus MHC restricted help (Julius *et al*, 1982; Zubler and Kanagawa, 1982; Layton *et al*, 1985; Tony and Parker, 1985) or a TI antigen (Thompson *et al*, 1984a) to stimulate entry into the cell cycle and responsiveness to T cell derived differentiation factors. A proportion of large B cells secrete Ig in response to nonspecific T cell factors in the absence of antigen (Noelle *et al*, 1983; Thompson *et al*, 1984a; Layton *et al*, 1985). Thus, the B cell requirement for various signals depends upon its activational state as reflected by cell size.

Evidence for functionally, phenotypically and genetically distinct B cell subpopulations comes from studies of CBA/N mice with the X-linked immunodeficiency (*xid*). TI pneumococcal (Amsbaugh *et al*, 1972) and other polysaccharide antigens (designated as type 2-TI, TI-2 antigens) as well as anti-Ig (Sieckmann *et al*, 1978), fail to induce antibody responses in these mice (reviewed in Mosier *et al*, 1977; Mosier and Subbarrao, 1982). Type 1-TI (TI-1) antigens do, however, stimulate antibody production in *xid* mice (Mosier *et al*, 1976; Mond *et al*, 1978). Recently, however, the validity of this classification has been challenged (Nossal and Pike, 1984). Ahmed *et al* (1977) described a series of B cell antigens, Lyb-3, 5, and 7, which are present on approximately 50% of B cells from normal mice but are absent from CBA/N B cells (reviewed in Kung *et al*, 1983). Thus, the immunodeficiency in CBA/N mice is at least partially due to the absence of a particular B cell subset. The Lyb-5^+ subset in normal mice is not detected until several weeks after birth, concomitant with responsiveness to TI-2 antigens (Kung *et al*, 1983), suggesting that the Lyb-5^- subset matures into the Lyb-5^+ subset. However, ther possibility that the two subsets represent separate lineages arising from distinct primitive precursors has not been ruled out.

The activation requirements of Lyb-5^+ and Lyb-5^- B cells have been extensively investigated by Singer and colleagues (reviewed in Singer *et al*, 1982; Howard and Paul, 1983; Singer and Hodes, 1983). TD responses by Lyb-5^+ B cells can be activated *via* both MHC restricted and MHC unrestricted pathways (Mosier and Feeney, 1984; Asano *et al*, 1985), in contrast to Lyb-5$^-$ B cells, which can only be activated in the presence of MHC restricted T cell help (Asano *et al*, 1981; Singer *et al*, 1981; reviewed in Singer *et al*, 1982). The nature of the help (MHC restricted *vs* unrestricted) delivered by a monoclonal T cell population was determined by antigen concentration (Asano *et al*, 1982). The exisitence of distinct B cell subpopulations which require different forms of T cell help appears to resolve earlier conflicting reports concerning the requirement for MHC restriction in T_h-B cell interactions (reviewed in Singer and Hodes, 1983).

B. Accessory Cells in B Cell Activation

Historical Perspectives

Only recently has it been appreciated that the phagocytic MØ, discovered over 100 years ago (Metchnikoff, 1884), plays such a pivotal, yet multifaceted role in the induction and regulation of specific immune responses. Aschoff (1924) demonstrated that a number of phagocytic cell types exist throughout the body, and suggested that they comprised one functional entity, which he termed the reticuloendothelial system (RES). Most early work stressed the importance of the RES in the nonspecific removal of foreign particles and antigens from blood and tissues (reviewed in Jaffé, 1931); however, the finding that experimental RES blockade decreased or abroagated humoral immunity suggested the importance of the RES in adaptive immunity. Indeed, MØ were at one point even thought to be the antibody producing cells (Sabin, 1923). Following Fagreus' demonstration (1948) that lymphocytes (plasma cells), and not MØ, synthesized antibodies, interest in the MØ and the RES as components of the adaptive immune system waned, until the provocative demonstration that antigen fed MØ produced a supraimmunogeinc antigen-ribonucleic acid complex (Fishman and Adler, 1963; Askonas and Rhodes, 1965). However, corroborating data in other systems was not forthcoming, and a revival of interest in MØ lymphocyte interactions in humoral immune responses awaited the cellular cooperation "revolution" of the late 1960's and 1970's.

The demonstration that *in vitro* antibody responses required MØ (Mosier, 1967; Pierce, 1969a), and that MØ associated antigen was more immunogenic than soluble antigen (Unanue and Askonas, 1968; Mitchison, 1969; Pierce *et al*, 1974) restored interest in the MØ as an important participant in specific immune responses. Lachmann (1971), Feldmann (1972b), Feldmann and Basten (1972, reviewed in Feldmann and Nossal, 1972; Basten and Mitchell, 1976) proposed that antigen specific T cell factors bound to the MØ surface would present antigens in a multivalent array or matrix thus enhancing their immunogencity. However, evidence for direct MØ-B cell interactions was difficult to obtain in most early systems, since they were TD.

More direct evidence that MØ, or other adherent accessory (A) cells, are directly involved in B cell responses, apart from their role in T cell activation, was provided by observations that MØ were required for antigen specific responses to TI antigens (Chused *et al*, 1976; Lee *et al*, 1976; Nordin, 1978; Boswell *et al*, 1980c; Letvin *et al*, 1981; Morrisey *et al*, 1981; Melchers and Lernhardt, 1985; Diner *et al*, 1986), as well as polyclonal responses to anti-Ig (Mongini *et al*, 1978) and mitogens (Persson *et al*, 1977; Martinez-Alonso *et al*, 1980; Fernandez and Palacios, 1982; Haeffner-Cavaillon *et al*, 1982; Melchers *et al*, 1982; Bandeira *et al*, 1983; Fernandez and Severinson, 1983). However, this matter remains controversial. Several early studies concluded that B cell responses to some TI antigens were A cell independent (Shortman *et al*, 1970; Feldmann and Palmer, 1971; Shortman and Palmer, 1971; Feldmann, 1972b; Rowley *et al*, 1973; Coutinho and Möller, 1975; Lipsky and Rosenthal, 1976; Rosenstreich and Oppenheim, 1976; Ishizaka *et al*, 1977; Kurland *et al*, 1977; Wong and Herscowitz, 1979; Boswell *et al*, 1980c; Morisaki *et al*, 1983). In some cases, MØ or their secreted products were shown to suppress B cell activation by TI antigens or mitogens (Diener *et al*, 1970; Yoshinaga *et al*, 1972; Lemke *et al*, 1975; Lipsky and Rosenthal, 1976; Kurland *et al*, 1977). These apparently contradictory results may be partially reconciled by the finding that MØ were stimulatory under suboptimal conditions of B cell activation and inhibitory (by an indomethacin sensitive mechanism) under supraoptimal conditions (Kurland *et al*, 1977). Furthermore, LPS, used to activate B cells in many of these studies, can modulate the production of stimulatory and inhibitory factors from MØ (Kurland *et al*, 1977; Mizel *et al*, 1978a). Thus, slight variations in culture conditions could result in the observation of different effects upon the addition of A cells, and the direct effects of mitogens on B cells must be distinguished from their indirect effects mediated through residual MØ.

Early hypotheses concerning MØ or A cell functions in TD antibody responses proposed that MØ or A cells processed antigens to render them immunogenic for T cells (Mosier, 1967; Shortman *et al*, 1970; Pierce *et al*, 1974; reviewed in Unanue, 1972). However, demonstrations that 2-mercaptoethanol (2-ME) could replace MØ function in some B cell systems (Chen and Hirsch, 1972; Lemke and Opitz, 1976; Nordin, 1978) appeared inconsistent with an antigen presentation function for MØ (Pierce *et al*, 1974; reviewed in Pierce and Kapp, 1976a). Pierce *et al* (1974) resolved this conflict by demonstrating that MØ have both viability promoting functions and antigen processing functions in TD B cell activation. Furthermore, they emphasized the difficulty of completely depleting MØ from lymphoid cell populations. Thus, 2-ME or MØ supernatants could restore the viability promoting requirement if residual MØ were present to fulfill the processing and presentation requirement (Pierce and Kapp, 1976a). In the case of TI antigens, a requirement for antigen presentation has never been demonstrated, thus, secreted MØ products may be sufficient to promote TI B cell responses in MØ depleted spleen cells.

A large body of work suggests that, in addition to antigen presentation and viability promotion, A cells secrete lymphokines required for optimal B cell activation by both TD and TI antigens. Thus, A cell function in B cell responses to TI antigens, mitogens, or anti-Ig could be replaced with secreted A cell products (Möller *et al*, 1976b; Kurland *et al*, 1977; Lee and Berry, 1977; Nordin, 1978; Corbel and Melchers, 1983). More recent studies have ascribed this activity to IL-1 (Booth *et al*, 1983; Falkoff *et al*, 1983; Howard *et al*, 1983b).

IL-1 was originally described as an A cell derived potentiating factor active in T cell mitogenic stimulation (Gery *et al*, 1972; Gery and Waksman, 1972), but recently, many cellular sources and targets of IL-1 activity have been identified (reviewed in Durum *et al*, 1985). The idea that soluble A cell products such as IL-1 may be involved in B cell activation is not a new one. Several groups have reported that A cell derived factors or purified IL-1 enhanced murine antibody responses in homozygous nude (*nu/nu*) or T depleted spleen cells to TD antigens *in vitro* (Hoffmann and Dutton, 1971; Schrader, 1973b; Wood and Gaul, 1974; Calderon *et al*, 1975; Wood and Cameron, 1976; Wood *et al*, 1976; Farrar *et al*, 1977; Koopman *et al*, 1978; Farrar and Koopman, 1979; Hoffmann and Watson, 1979; Hoffmann *et al*, 1979; Wood, 1979; Hoffmann, 1980; Schrader and Nossal, 1980), as well as human B cell stimulation by anti-μ and mitogens (Oppenheim *et al*, 1980; Rosenberg and Lipsky, 1981; Falkoff *et al*, 1983; Lipsky *et al*, 1983; Kehrl *et al*, 1984). Despite the relative T cell independence of such responses, it is difficult to assert that IL-1 acts directly on B cells. Indeed, it has been suggested (Farrar *et al*, 1982) that the IL-1 effect on antibody responses is mediated only through T cells. Nevertheless, considerable evidence has recently accumulated supporting a direct role for IL-1 during several stages of B cell differentiation.

The earliest stage of B cell maturation at which IL-1 has been reported to act is during the pre-B→B cell transition (Giri *et al*, 1984). IL-1 has been further observed to act during antigen, mitogen, or anti-Ig stimulated proliferation and differentiation. Hoffmann (1980) reported that exposure of B cells to IL-1 increased expression of Ia, complement receptors, and mIg, as well as the frequency of responsive B cells. Several groups have suggested that IL-1 acts early in the B cell response, during the proliferative phase (Farrar *et al*, 1977, 1980; Falkoff *et al*, 1983; Howard and Paul, 1983; Booth and Watson, 1984; Corbel and Melchers, 1984; Melchers and Lernhardt, 1985). Others find that IL-1 can act as a differentiative factor, either alone (Pike and Nossal, 1985a), or synergistically with BCDF (Kehrl *et al*, 1984). Most investigators find that IL-1 does not act on resting B cells (Falkoff *et al*, 1983; Howard and Paul, 1983), however, Booth *et al* (1983) found that IL-1 induced the differentiation of some B cells in the absence of other activating stimuli.

In most reports, IL-1 was found to act synergistically with anti-μ (Booth *et al*, 1983; Falkoff *et al*, 1983; Kehrl *et al*, 1984), or other growth factors such as BCGF (Howard *et al*, 1983b), TRF (Farrar *et al*, 1977; Farrar and Koopman, 1979; Hoffmann and Watson, 1979), IL-2 plus γ-interferon (Leibson *et al*, 1982, Kappler *et al*, 1983) or IL-2 (Mond, 1982). In some systems, IL-1 merely enhanced the AFC response in A cell and T cell depleted spleen cells (Kappler *et al*, 1983; Leibson *et al*, 1982), whereas in others, there was an absolute IL-1 requirement (Howard *et al*, 1983). This may have been due to incomplete MØ depletion in the former case. The requirement for IL-1 may also depend upon the strength of the initial activating signal. Thus, Falkoff *et al* (1983) observed an IL-1 requirement for human B cells only when anti-μ stimulation was suboptimal and BCGF was absent.

The best evidence that IL-1 acts directly on B cells comes from Pike and Nossal (1985a). Single, fluorescein (FLU) specific B cells were stimulated by FLU-ficoil, a TI antigen, to proliferate and secrete Ig in response to IL-1 alone, or IL-1 together with IL-2. Contrary to Howard and Paul's report (1983) that IL-1 acts in late G_1, Pike and Nossal concluded that IL-1 acts during all phases of B cell activation – induction (to promote the $G_0 \rightarrow G_1$ transition), proliferation, and differentiation. However, regardless of the point of action of IL-1, Pike and Nossal's observation, coupled with the recent description of IL-1 receptors on activated B cells (Dower *et al*, 1985), strongly suggest that A cell derived IL-1 can have direct effects on B cell growth and differentiation.

MHC Restriciton of A Cell-B Cell Interactions

A cell-B cell cooperation may, in some cases, be mediated through cell-cell contact, in addition to A cell derived factors, such as IL-1. Descriptions of MHC restricted A cell-B cell interactions have raised the possibility that, contrary to prevailing opinions, the recognition specificities of T and B cells are not fundamentally different. Katz *et al* (1978) first suggested that B cells could distinguish between T_h of different MHC haplotypes, and that this preference was determined not by the B cell genotype, but by the MHC environment in which the B cells had differentiated. Howie and Feldmann (1978) demonstrated an immune response (*Ir*) gene effect operating at the level of the A cell-B cell interaction, since nonresponder MØ could not present antigen specific helper factor to responder B cells. Based on this observation, they suggested that genetic restrictions may exist between A cells and B cells.

Gorczynski *et al* (1980) utilized a TD system, in which the helper signal was provided by LPS to remove the potentially obscuring influence of the MHC restricted T_h-B cell interaction, to provide more direct evidence of A cell-B cell restriciton. They observed that F_1 B cells primed in an irradiated parental host could be restimulated *in vitro* most efficiently by antigen pulsed A cells syngeneic to the priming host. Such results suggested that B cells recognize antigen in association with Ia on the surface of A cells, analogous to T_h recognition.

Nisbet-Brown *et al* (1981) derived a similar conclusion from a different experimental approach in which primed, mutually tolerant T and B cells from one-way parent→F_1 fetal liver chimeras were restimulated in an irradiated normal or chimeric host. In contrast to other reports, neither T_h-A cell nor T_h-B cell restriction was observed. Nevertheless, B cell stimulation was optimal in syngeneic hosts. The results were interpreted in favor of MHC restricted A cell-B cell cooperation, which may have been evident only because of the unusual lack of T_h-B restriction. However, an alternative explanation, namely, that there was T_h-A cell restriction during the priming in the F_1 host and T_h-B cell restriction during the restimulation, could not be completely ruled out.

Singer and Hodes (1982) and Hodes *et al* (1983a,b) assessed the possibility of A cell-B cell restrictions in TI and TD responses to trinitrophenyl (TNP)-ficoll, respectively. Under TI conditions, B cells from F_1→parent semiallogeneic or A→B fully allogeneic chimeras cooperated only with host type A cells. This could not be explained on the basis of MHC restricted T_h-A cell interactions, since intentional addition of appropriate T_h failed to alter the observed restriction patterns. Furthermore, chimeric A→B B cells were not activated when simultaneously present during activation of strain A B cells in the presence of strain A T_h and A cells.

When B cells were cultured with limiting concentrations of TNP-ficoll, the response was shown to be highly TD. Under these conditions, T_h and B cells were required to recognize self MHC on A cells, but not on each other. Once again, A cell-B cell restriction could only be observed in the apparent absence of T_h-B cell restriction. Since the addition of soluble TRF abrogated the A cell requirement and obscured the requirement for B cell recognition of A cell

MHC molecules, it was suggested that, in contrast to T_h activated by classical TD antigens, TNP-ficoll responsive T_h secrete minimal amounts of TRF and may represent a distinct T_h subset. Thus, if A cell-B cell restriction existed in TD responses, it would be obscured by TRF. Although not considered by the authors, perhaps A cell-B cell restriction in the TNP-ficoll response serves to bring T_h and B cells close enough together for the limiting amount of TRF secreted by TNP-ficoll responsive T_h to exert its effects.

In summary, although several studies suggest the existence of MHC restricted A cell-B cell interactions, the use of high cell densities for *in vitro* studies (Gorczynski *et al*, 1980), or complicated protocols involving chimeric B and T cells *in vivo* (Nisbet-Brown *et al*, 1981), or *in vitro* (Singer and Hodes, 1982; Hodes *et al*, 1983a,b), raise the possibility that the observed restriction patterns reflect underlying T_h-A cell interactions. While reports of MHC restricted antibody (van Leeuwen *et al*, 1979; Wylie *et al*, 1982) appear to lend credence to the above reports, it is not clear whether such MHC restricted recognition has the same relevance for B cell activation as it does for T cell activation.

C. B Cell Tolerance

The ability to discriminate between self and nonself antigens is vital to the functioning of the adaptive immune system as a specific defense against pathogenic microorganisms, viruses, and neoplasms. Failure of the immune system to "tolerate" self tissues can result in pathological autoimmune states leading to severe debilitation and sometimes death. Thus, the process by which the immune system acquires a definition of self is crucial in ensuring that the immune artillery is directed only at foreign invaders.

As the complexity of cellular interactions involved in normal immune responses has been revealed in the years since the discovery of separate B and T lymphocyte lineages (Claman *et al*, 1966; Miller and Mitchell, 1967), it has become obvious that the lymphocyte-antigen interactions leading to a state of unresponsiveness are unlikely to be the result of a single all-encompassing mechanism. Moreover, the diversity of form (eg., circulating soluble *vs* cell bound), concentration (from femtomolar or picomolar to millimolar), and valency (monovalent, paucivalent, and multivalent) of self antigens likely necessitates distinct modes of silencing autoreactive lymphocyte clones. I shall therefore adopt a pluralistic view in summarizing the literature concerning the establishment and maintenance of self tolerance in the B lymphocyte lineage.

Historical Perspectives

Paul Ehrlich, upon observing that antibodies were produced only in response to foreign erythrocytes, was the first to conceptualize that the immune system must have evolved a mechanism to avoid *horror autotoxicus* (Ehrlich and Morgenroth, 1900). Several decades passed, however, before the problem of self-nonself discrimination was directly approached on an experimental and theoretical basis. One important breakthrough came from experiments demonstrating that an individual's response to an antigen could be altered by an encounter with that antigen very early in life. In 1938, Traub observed that mice infected with lymphocytic choriomeningitis virus *in utero* harbored the virus in various tissues throughout life, unable to generate an immune response against it, whereas mice infected as adults mounted vigorous anti-viral responses. Similarly, Owen observed that dizygotic cattle twins, which shared a common placenta, had chimeric hemopoietic systems and exhibited "tolerance" to each other's cells throughout life (Owen, 1945, 1956). These observations contributed to Burnet and Fenner's 1949 proposal that antigens encountered during embryonic life, prior to the development of immunocompetence, would be considered self antigens and therefore would not induce immunity when encountered later in life. This clonal deletion concept was incorporated into Burnet's Clonal Selection Theory, which provided a comprehensive theoretical framework for both immune induction and the establishment of self tolerance (Burnet, 1957, 1959).

Burnet's clonal deletion theory of immunologic tolerance proposes that the immune system defines self antigens as those which it encounters during a specific ontogenetic phase of development. Lymphocytes bearing receptors for such antigens are "forbidden clones" to be somehow deleted, thus preventing autoimmunity. Lederberg further suggested that the tolerance sensitive phase would occur during the ontogeny of the lymphocyte, rather than that of the individual (Lederberg, 1959). If a lymphocyte matured beyond this stage (ie., had not been deleted by encounter with a self antigen), it would become inducible. This modification, later termed clonal abortion by Nossal and Pike (1975a), accounted for the need to delete autoreactive clones arising in adult life from the continually recycling lymphocyte population.

The ability to induce tolerance experimentally to extrinsic (or foreign) antigens was a major technical advance first accomplished in the mouse by Billingam, Brent and Medawar (1953), and independently, in the chicken, by Hašek (1954). Both groups demonstrated that living allogeneic cells administered to a fetus or to a very young neonate (Billingham *et al*, 1956), specifically impaired skin graft rejection of the donor strain. These results conformed to the predictions of the clonal deletion theory. That the immature animal is more easily tolerized by xenogenic serum protein antigens (Hanan and Oyana, 1954; Cinader and Dubert, 1955; Dixon and Maurer, 1955; Smith and Bridges, 1958) and erythrocytes (Nossal, 1958) provided further validation of this concept.

By the mid to late 1960's, the phenomenon of immunologic tolerance was firmly ensconced in the immunological literature (reviewed by Dresser and Mitchison, 1968) and was generally thought to be the result of one all-encompassing mechanism. However, the demonstration that T cell tolerance could secondarily result in B cell unresponsivenss (Taylor, 1969; Chiller *et al*, 1971; Weigle, 1971), and the discovery of suppressor T cells (T_s) (Gershon and Kondo, 1970, 1971; McCullagh, 1970), suggested a need to re-evaluate mechanisms of immunological tolerance.

Subsequently, two other mechanisms, in addition to clonal deletion, were invoked to explain self tolerance in the B lymphocyte lineage - receptor or effector cell blockade, and active suppression by T_s. As the clonal deletion/abortion literature is the most extensive it will be reviewed first, followed by a discussion of receptor blockade and active suppression. The functions of MØ in B cell tolerance, including those which may pertain to the above mechanisms, will be discussed separately.

Models for B Cell Tolerance

Deletional Models

The most fundamental tenet of the clonal abortion hypothesis is the differential sensitivity of immature *vs* mature B cells to negative signalling by antigen (Lederberg, 1959; Nossal and Pike, 1975a). The first support for this concept came from studies of anti-immunoglobulin (Lawton *et al*, 1972; Manning and Jutila, 1972a,b) and anti-idiotype (id) induced (Köhler *et al*, 1974; Strayer *et al*, 1974; Accolla *et al*, 1977) suppression of immune responses. Prolonged treatment either *in vivo* (reviewed by Lawton and Cooper, 1974), or *in vitro* (Raff *et al*, 1975; Sidman and Unanue, 1975; Bruyns *et al*, 1976; Kearney *et al*, 1976) with anti-Ig reagents produced profound, irreversible unresponsiveness in immature B cells. In contrast, unresponsiveness in adult cells was of shorter duration and reversible (Strayer *et al*, 1974; Raff *et al*, 1975; Sidman and Unanue, 1975). The profound immunosuppression in anti-Ig treated neonatal mice was the result of arrested differentiation of mIg^- pre-B cells to mIg^+ B cells in lymphoid organs (Lawton and Cooper, 1974; Raff *et al*, 1975) and was not attributable to T_s (Manning and Jutila, 1972a,b). Immature B cells were rendered unresponsive by much lower concentrations of anti-Ig than were adult cells (Raff *et al*, 1975; Kearney *et al*, 1976). Such hypersusceptibility of immature B cells to negative signalling by anti-Ig was not due to intrinsically slower rates of synthesis of new Ig receptors, since pronase treated immature and mature B cells regained new receptors with the same kinetics (Sidman and Unanue, 1975). Furthermore, unresponsiveness was not caused by receptor blockade, since pronase treatment following anti-Ig treatment did not restore responsiveness (Sidman and Unanue, 1975).

The heightened susceptibility of immature B cells to negative signalling was extended to antigen specific B cells by Nossal and Pike (1975b), and subsequently confirmed by several groups (Cambier *et al*, 1976; Metcalf and Klinman, 1976; Elson, 1977; Stocker, 1977; Venkataraman and Scott, 1977; Waters *et al*, 1979). Tolerance in immature B cells was generally achieved by much lower concentrations of tolerogen than those required to tolerize adult B cells (Cambier *et al*, 1976; Stocker, 1977; Nossal and Pike, 1978); Metcalf *et al*, 1979;

Kay *et al*, 1980). B cells exposed to either hapten-human gamma globulin (HGG) (Nossal and Pike, 1978; Pike and Nossal, 1979; Pike *et al*, 1980) or anti-μ (Nossal *et al*, 1979; Pike *et al*, 1982) as tolerogens during the pre-B→B cell transition (ie., during the acquisition of mIg receptors) exhibited the most profound susceptibility to tolerance induction.

Nossal and Pike described a gradual acquisition of resistance to tolerance induction as B cells matured from the pre-B, to immature (eg., B cells from neonatal mice), to the adult stage. The threshold tolerogen concentration required to induce tolerance during the pre-B→B transition was approximately 1000-fold lower than the threshold concentration for immature B cells, which in turn was about 1000-fold lower than that for adult B cells (Nossal and Pike, 1978; Pike *et al*, 1980; reviewed in Nossal, 1983). Consistent with this, Metcalf and Klinman (1977) demonstrated that susceptibility to tolerance induction could serve as a general marker for B cell immaturity. Over 90% of splenic dinitrophenyl (DNP) specific B cell clones from 1 day old neonates were susceptible to tolerance induction, with the proportion of tolerance resistant clones increasing to adult levels by 7 days after birth. However, adult levels of tolerance resistance in bone marrow B cells were not acheived until much later (6-8 wks alfter birth). Twenty-five percent of adult bone marrow cells remained susceptible to tolerance induction, probably reflecting the existence of the generative B cell pool in the marrow (reviewed in Metcalf *et al*, 1979).

A further refinement to the clonal abortion theory was proposed by Vitetta and Uhr (1975), who suggested that the increased resistance to tolerance induction that occurred during B cell maturation was a consequence of the acquisition of mIgD. This hypothesis was based on the observations that 1) most B cells in fetal and neonatal animals are mIgM$^+$ mIgD$^-$ and 2) the concomitant acquisition of functional competence and mIgD in 1 wk old neonatal mice (reviewed in Kettman *et al*, 1979). Accordingly, enzymatic removal (Cambier *et al*, 1977a; Vitetta *et al*, 1977) or antibody blocking of surface IgD (Scott *et al*, 1977; Zitron *et al*, 1977) restored tolerance susceptibility in mature TD B cells. It was proposed that surface IgD transmits a positive triggering signal, whereas mIgM transmits a negative signal to TD B cells (reviewed by Kettman *et al*, 1979). However, contrary to the suggested function of mIgD as a determinant of tolerance resistance, others have shown that separated populations of IgD^+ and IgD^- B cells were equally tolerizable when assessed by either TI (Layton *et al*, 1979a) or TD (Layton *et al*, 1979b) challenge. Moreover, loss of tolerance susceptibility may occur before IgD is detectable in ontogeny (Metcalf and Klinman, 1976). Thus, the function of IgD in tolerance is still unresolved.

Signal Discrimination Models

The Two Signal Theory

Bretscher and Cohn (1968, 1970; Cohn, 1972) proposed that, in contradistinction to the clonal abortion theory of self tolerance, antigen specific B cells remained both paralyzable and inducible by antigen throughout their lifetime. In this case, the outcome (immunity *vs* tolerance) of an antigen-Ig receptor interaction, termed Signal 1, would be determined by the presence or absence of carrier specific, T cell derived helper factors (Signal 2 or associative antibody) for which the B cell was postulated to have separate receptors. In theory, both Signal 1 and Signal 2 would result in B cell triggering; Signal 1 alone would paralyze. This theory placed the burden of self tolerance at the level of the T cell. During ontogeny, B cells were thought to arise prior to T cells; thus, only Signal 1 would be available, resulting in paralysis of all B cells specific for self antigens present at this time. Autoreactive B cells arising later in life would be paralyzed by the lack of T cell help for such antigens. The induction and maintenance of self tolerance in the T cell compartment would thus be critical, and was later proposed to occur by a similar mechanism (Cohn and Epstein, 1978). The two signal theory can be considered a more refined version of Burnet's Clonal Deletion Theory, since the absence of Signal 2 during B cell ontogeny would result in a tolerance susceptible phase early in the development of the animal. However, contrary to the clonal abortion dogma, the environment (paucity of Signal 2) rather than the intrinsic nature of the B cell, would be the determining factor.

Consistent with the Bretscher-Cohn hypothesis, the presence of Signal 2, in the form of antigen specific T cell help, or Signal 2 replacing TI mitogens, such as LPS, can interfere with tolerance induction (Katz *et al*, 1971; Louis *et al*, 1973a; Schrader, 1975a; Metcalf and Klinman, 1977; Pike and Nossal, 1979; Teale *et al*, 1979; Diener *et al*, 1982). However, resistance to tolerance induction in the presence of LPS was only about 30% effective with HGG as a tolerogen (Pike and Nossal, 1979), and not at all effective for polymerized flagellin (POL) induced tolerance (Scott and Diener, 1976). This could reflect heterogeneity in B cell subsets responsive to LPS (Gronowicz and Coutinho, 1974; McKearn *et al*, 1982) or a requirement for an alternative form of Signal 2 by those B cells not rescued from tolerance induction. Thus, the usefulness of the two signal model as an explanation for self tolerance appears limited.

One Signal Model

The one nonspecific signal model (Coutinho and Möller, 1974, Coutinho, 1975; Möller, 1975; Coutinho *et al*, 1984) views the antigen specific Ig receptor on B cells as merely a passive focussing device, rather that a signal delivery device. B cell activation is proposed to occur as a consequence of interactions between the carrier, which possesses polyclonal B cell activating (PBA) properties, and non-Ig PBA receptors on the B cell, hence one nonspecific signal. This idea was based on the observation that many TI antigens have PBA properties (Coutinho and Möller, 1973). At low concentrations, hapten-TI antigen triggers only hapten specific B cells, due to the focussing of hapten-TI complexes onto hapten specific B cells, whereas at high concentrations of hapten-TI, polyclonal activation takes place. B cell tolerance was postulated to be the result of overstimulation by PBA, since the high concentrations of hapten-TI which polyclonally activated most B cells inhibited the hapten specific response (Coutinho and Möller, 1974). It was further postulated that B cell tolerance was only necessary for self antigens possessing PBA properties. Tolerance to the majority of antigens, which are TD, would be maintained in the T cell compartment.

There are several major conceptual and experimental objections to this model (Cohn and Blomberg, 1975). B cells can be rendered tolerant to TD antigens, and the interaction of mIg can deliver a positive or negative signal. Moreover, tolerance can be induced to completely nonimmunogenic hapten-carbohydrate conjugates exhibiting no PBA activity over a wide range of concentration (von Borstel *et al*, 1983). More recently, the binding of TD antigens or anti-Ig to B cells was demonstrated to cause transition from the G_0 phase of the cell cycle to a "poised" state, with further progression through G_1 and S phases being dependent upon MHC restricted help and T cell derived nonspecific factors (reviewed in Cambier *et al*, 1985). Thus, the Ig receptor is capable of signal transmission, contrary to the major premise of this model.

Mechanisms of Clonal Deletion/Abortion Tolerogenesis

As the name suggests, deletional theories of self tolerance propose that autoreactive B cell clones are deleted following contact with antigen during a tolerance susceptible phase of development. Therefore, in its narrowest interpretation, the theory would predict that B cell clones capable of binding antigens to which an animal is tolerant should not exist. Consequently, many early studies, using tolerance induction to extrinsic antigens as models for self tolerance, compared the numbers of antigen binding cells (ABC) in normal, immunized, and tolerant animals. Tolerant animals were reported to have normal (Ada, 1970; Ada *et al*, 1970; Humphrey and Keller, 1970; Cooper *et al*, 1972), increased (Howard and Siskind, 1969; Sjöberg, 1970; Möller and Sjöberg, 1972), or decreased (Naor and Sulitzeanu, 1969; Katz *et al*, 1971; Louis *et al*, 1973b; Aldo-Benson and Borel, 1974; Bruyns *et al*, 1976; Venkataraman and Scott, 1977), or slightly decreased (Nossal and Pike, 1980) ABC, depending upon the particular antigen used. A deletional mechanism was indicated for anti-id induced B cell tolerance, since neonatal mice injected with anti-id lacked id$^+$ B cell clones, whereas adult mice so treated had id$^+$ clones which were only reversibly blocked (Köhler *et al*, 1974; Accolla *et al*, 1977). It appears therefore, that the physical deletion of B cells as a mechanism of unresponsiveness can only be demonstrated with particular tolerogens.

The possibility remains that B cells are functionally silenced without being physically deleted. Interference with any step of the cyclical process of receptor modulation and re-expression following antigen binding could theoretically cause functional inactivation of the B cell. Diener and Paetkau's (1972) observation that tolerogenic concentrations of POL inhibited capping and endocytosis, resulting in a "frozen" B cell, at first appeared to provide a biophysical basis for B cell tolerance; however, it was subsequently demonstrated that inhibition of capping was unrelated to the tolerance phenomenon (Diener *et al*, 1976; Sidman and Unanue, 1976). Tolerogenic signals were thus presumed to be transmitted across the membrane.

Some evidence exists that B cell tolerance results from interference with a later stage of the cycle – the inhibition of Ig receptor re-expression following capping and endocytosis. Immature B cells were shown unable to regain their mIg receptor coat following modulation with anti-Ig, whereas mature B cells retained this capacity (Sidman and Unanue, 1975; Bruyns *et al*, 1976). Furthermore, tolerogenic concentrations of some antigens also inhibit receptor re-expression (Ault *et al*, 1974; Nossal and Layton, 1976; Klaus *et al*, 1977; Bankert *et al*, 1978). However, Nossal and Pike (1980) detected, in mice neonatally tolerized to FLU-HGG, significant numbers of FLU binding B cells which were incapable of responding to antigen or mitogen. Similarly, Pike *et al* (1982) more recently demonstrated that certain concentrations of a monoclonal anti-μ antibody, which failed to inhibit the acquisition of mIg by pre-B cells, nonetheless induced profound B cell unresponsiveness. Such B cells were functionally "anergic". Despite mIg densities similar to untreated controls, anergic B cells failed to proliferate or secrete Ig in response to a powerful polyclonal activating stimulus. Moreover, Diener *et al* (1976) found that B cells treated with supraimmunogenic concentrations of POL remained unresponsive despite regeneration of Ig receptors. The existence of anergic B cells perhaps reconciles old observations concerning the coexistence of self tolerance with B cells capable of binding self antigens (Bankhurst *et al*, 1973; Roberts *et al*, 1973; Yung *et al*, 1973; reviewed by Elson *et al*, 1977). Self reactive B cells can, however, be activated by LPS and other polyclonal activators *in vivo* (Fournée *et al*, 1974) and *in vitro* (Hammarstrom *et al*, 1976; Primi *et al*, 1977). Whether such B cells are truly anergic, and can only be triggered under nonphysiological conditions, or whether they are under active suppression, remains to be determined.

While not shedding light on the fate of neonatally tolerized B cells, studies by Teale and Klinman (1980, 1984) using inhibitors of various cellular functions, have elucidated some of the metabolic requirements for the induction of tolerance. They conclude that the induction of B cell tolerance is an active process, requiring DNA, RNA and protein biosynthesis, energy generation, and a methyltransferase activity, but not cytoskeleton functions. Diener *et al* (1976) and Boyd and Schrader (1982) confirmed that colchicine sensitive cytoskeletal functions are not involved in transmission of negative signals to adult B cells, whereas Desaymard (1981) reached the opposite conclusion. Carrier related differences in the mechanisms of tolerance induction may account for this discrepancy.

Evidence Against Clonal Deletion/Abortion

The clonal abortion and deletion theories designate the epitope-Ig receptor interaction as the only relevant interaction leading to tolerance induction in susceptible B cells. Numerous studies, showing a correlation between epitope density and tolerogenic potential (Feldmann, 1972c; Desaymard and Feldmann, 1975; Desaymard *et al*, 1976; Pike *et al*, 1981; Waldschmidt *et al*, 1983, Waldschmidt and Vitetta, 1985; reviewed in Diener and Feldmann, 1972a; Feldmann *et al*, 1975), suggested that the avidity of interaction between the epitope and Ig receptor is the most critical parameter in determining tolerogenicity of antigens. It was further suggested that the delivery of too many signals, as a result of cross-linking beyond a critical threshold, induces an unresponsive state in the B cell (Diener and Feldmann, 1972b). Such a conclusion is strengthened by observations concerning the greater ease of tolerance induction by multivalent *vs* monovalent antigens (Metcalf and Klinman, 1976; Elson, 1977; Szewczuk and Siskind, 1977), and the ability of high avidity IgG precursors to be tolerized at much lower epitope densities and at lower concentrations than low avidity IgM precursors (Desaymard *et al*, 1976).

Recent studies challenge the concept that only the eptiope-Ig interaction is relevant. The immunogenic or tolerogenic potential of DNP conjugated to various polymeric carbohydrate carriers, all displaying equal hapten density and antibody binding avidity, depended solely on the carrier moiety (Diener *et al*, 1981). In some cases, the nontolerogenic conjugates displayed higher antibody binding avidity than the tolerogenic conjugates (von Borstel *et al*, 1983). Thus, in contradistinction to the predictions of both clonal abortion and deletion, factors other than the avidity of epitope-Ig receptor interaction occurring on a tolerance susceptible B cell determine the tolerogenic potential of a hapten-carrier conjugate.

Gamma Globulin Carriers

It has been apparent for some time that the nature of the carrier significantly affects the tolerogenic potential of hapten-carrier conjugates. Nonimmunogenic, or poorly immunogenic, carriers, such as autologous serum proteins (Havas, 1969; Borel, 1971; Golan and Borel, 1971), the capsular polypeptide of *Bacillus anthracis* (Roelants and Goodman, 1970), and poly-D-(G,L) (Katz *et al*, 1971) are among the most potent tolerogens, possibly due to a lack of T cell help, in accordance with the Bretscher-Cohn two signal hypothesis (1968, 1970). Gamma globulins (reviewed in Scott *et al*, 1979; Nossal, 1983), particularly isologous IgG (reviewed in Borel, 1976), are potent tolerogens. Most of the literature supporting clonal abortion as the mechanism of B cell tolerance involves the use of gamma globulins as a carrier for the induction of hapten specific tolerance.

The potent suppressive or tolerogenic effect of gamma globulins depends primarily on the presence of the Fc piece; F(ab) or F(ab')$_2$ fragments are usually, but not always (Feldmann and Diener, 1972; Schrader, 1975b), much less effective. Pike *et al* (1981) compared the efficacy of FLU conjugates of HGG, F(ab')$_2$ fragments of HGG, or bovine serum albumin (BSA) with various hapten densities, in tolerizing neonatal B cells. At each hapten substitution ratio, intact HGG was the most effective carrier, underscoring the importance of the Fc piece. The differences in tolerogenic capacity of the various carriers could be compensated for by increasing the hapten density. Similarly, Waldschmidt *et al* (1983) used F(ab')$_2$ conjugates with higher hapten densities to increase their tolerogenic capacity for adult B cells. At higher hapten densities, immature B cells were more susceptible to tolerization than mature B cells, regardless of the presence of the Fc piece (Waldschmidt and Vitetta, 1985). However, at lower hapten densities, Fc containing tolerogens were superior. The inactivation of B cells by anti-Ig is also Fc dependent (Sidman and Unanue, 1976; Köhler *et al*, 1977; Tony and Schimpl, 1980; Phillips and Parker, 1983, 1984). All of these results suggest that negative signals are generated by processes other than, or in addition to, (Phillips and Parker, 1984) the cross-linking of mIg receptors on the B cell.

Antibody or immune complex mediated suppression of antibody responses was first demonstrated by Smith (1909) and is now well established (Uhr and Möller, 1968; Diener and Feldmann, 1970, 1972b). The Fc dependence of this phenomenon has also been demonstrated (Sinclair, 1969, 1983; Köhler *et al*, 1977; Oberbarnscheidt and Kölsch, 1978; Taylor *et al*, 1979; Tite and Taylor, 1979; Nelson and Manning, 1981). Since B cells bear Fc receptors (FcR) (Dickler, 1976), this possibly represents a normal homeostatic mechanism for regulating antiboldy production *via* negative feedback (Taylor *et al*, 1979; reviewed in Taylor, 1982; Sinclair, 1983). The facilitation of tolerance induction to normally immunogenic concentrations of antigen in the presence of low concentrations of specific antibody (Diener and Feldmann, 1970), or by covalent antigen-antibody complexes (Taylor *et al*, 1979; Tite and Taylor, 1979), may be a further manifestation of this homeostatic regulation. Tolerance induced by this means has been shown to be the result of "peripheral neutralization" of antigen, thus preventing access to immunocompetent cells (Uhr and Möller, 1968), or a "central effect", resulting in specific inactivation of the B cell (Diener and Feldmann, 1970), or both, depending on the relative concentrations of antigen and antibody (Diener and Feldmann, 1972b; Hoffmann and Kappler, 1978). Cumulatively, these observations suggest that the peculiar potency of gamma globulins as tolerogens results from an inherent sensitivity of the B cell to Fc dependent negative signalling by Ig, and may have very little to do with the establishment of self tolerance, except perhaps to Ig itself. Indeed, the enhanced tolerogenicity of multivalent hapten-gamma globulin compounds is perhaps due to a negative signal generated as a result of cross-linking both mIg and FcR simultaneously (Phillips and Parker, 1984). This negative signal may be mediated by the inhibition of inositol phospholipid breakdown (Bijsterbosch and Klaus, 1985), an early mIg transduced signal in B cell activation (Coggeshall and Cambier, 1984; Bijsterbosch *et al*, 1985).

Other Carriers

Diener *et al* (1981) and von Borstel *et al* (1983) evaluated the tolerogenic capacity of the completely nonimmunogenic compounds DNP-methylcellulose (MC) and DNP-carboxymethlycellulose (CMC) in their native or oxidized and reduced forms. DNP-MC and DNP-CMC induced profound, hapten specific unresponsivenss *in vitro* and *in vivo*, respectively, which was not due to active suppression or receptor blockade (Diner *et al*, 1979; Diener *et al*, 1981; von Borstel *et al*, 1983). Contrary to the predictions of clonal abortion/deletion theory, the oxidation and reduction of these compounds severely diminished thier tolerogenic capability (Diener *et al*, 1981; von Borstel *et al*, 1983). Interestingly, the tolerogenic capacity of high concentrations of DNP-dextran was also destroyed by this chemical modification, but the immunogenic capacity of lower concentrations was unaffected (Diener *et al*, 1981). Chemical alteration of DNP-MC did not significantly alter the molecular weight or conformation, as assessed by determining the sedimentation coefficient. Moreover, this nontolerogenic form displayed a higher avidity for DNP specific AFC than the native form. Thus, T cell independent tolerance to DNP-MC and DNP-CMC appears to be entirely dependent on the carrier moiety.

Experiments designed to introduce extrinsic antigens to the developing immune system, under conditions similar to those in which self antigens would be encountered *in vivo*, have further emphasized the role of the carrier in determining the outcome of an antigenic encounter early in ontogeny. Deaggregated HGG or bovine gamma globulin (BGG), introduced *in utero* by transplacental transfer *via* the mother, effected long lasting, HGG specific tolerance at both the T and B cell levels (Waters *et al*, 1979), the mechanism of which was consistent with clonal abortion. However, transplacental administration of BSA, $[\text{Glu-Tyr-Lys-(Glu-Tyr-Ala)}_5]_n$, and ovalbumin (OVA) failed to induce tolerance, despite the fact that after having crossed the placenta these antigens were maintained at concentrations similar to HGG (Diener and Waters, 1980). Similarly, the mechanisms of TNP specific B cell unresponsiveness induced by neonatal injection of TNP-HGG and TNP-BSA were distinct (Waters and Diener, 1983). TNP-HGG tolerized mice had decreased TNP specific AFC precursor frequencies as compared to uninjected littermates, consistent with a clonal abortion mechanism of tolerogenesis. In contrast, TNP-BSA tolerized mice had normal TNP specific AFC precursor frequencies; tolerance was due to active suppression by T cells.

In addition to determining which cellular mechanism of tolerance is operative in a given situation, the carrier may also select which B cell subpopulation (TD *vs* TI) is tolerized. Howard and Hale (1976) suggested that only TD B cells are tolerized by clonal abortion, since they observed no difference in sensitivity between neonatal and adult cells to tolerance induction by TI antigens. Similarly, Cambier *et al* (1977b) found that only the TD subset was significantly unresponsive in TNP-HGG induced neonatal tolerance. This apparently contradicts a report by Waters and Diener (1983) that neonatal tolerance induction to TNP-HGG resulted in specific unresponsivenss of both TD and TI subsets; however, mechanisitic differences between neonatal tolerance induced *in vitro* (Cambier *et al*, 1977b) and *in vivo* (Waters and Diener, 1983) may account for this discrepancy. Marshall-Clarke and Playfair (1979) also reported that certain tolerogens acted differentially on TD and TI B cell subsets.

Further carrier related differences in tolerance susceptibility have been revealed within the TD and TI B cell subsets. Only the IgG secreting TD subset was significantly unresponsive in mice rendered neonatally tolerant *in vivo* to BSA, whereas the IgM secreting TD subset displayed normal responsiveness; unresponsiveness at the level of the whole animal was due to active suppression (Waters and Diener, 1983). Differences in the tolerance susceptibility of B cells responsive to TI-1 and TI-2 antigens have also been reported (von Borstel *et al*, 1983). Mice neonatally treated with DNP-MC were hapten specifically unresponsive to the TI-2 antigen DNP-ficoll, but not to the TI-1 antigens DNP-*Brucella abortus* (Ba) or the mitogen LPS. Conversely, with TNP-OVA as the tolerogen, both the TD and TI-1 subsets were rendered unresponsive, whereas the TI-2-(TNP-ficoll) responsive subset was unaffected. Finally, in agreement with earlier studies (McKearn and Quintans, 1980), Waters and Diener (1983) observed that within the TI-1 responsive subset, Ba responsive cells were relatively more tolerance resistant than were LPS responsive cells.

The preceding discussion has provided extensive evidence that the simplest versions of the clonal abortion and deletion theories for B cell tolerance are not supported by experimental realities. The most serious objection relates to the well established influence of the carrier in most reports of hapten specific B cell tolerance. The differential sensitivity of immature B cells to negative signalling, which has provided the bulk of experimental support for clonal abortion, clearly depends heavily upon the special tolerogenic properites of gamma globulin carriers. Both mature and immature B cells are susceptible to Fc mediated negative signalling by Ig, a fact probably more relevant to homeostatic feedback regulation than to avoidance of autoimmunity. Why immature B cells should be more sensitive to such feedback regulation is not immediately apparent, but this phenomenon could be entirely unrelated to the elimination of autoreactive B cells during development.

In defense of clonal abortion/deletion are observations that B cells bearing receptors for antigens present in high concentrations in the serum, such as albumin, cannot be detected (Unanue, 1971; Bankhurst *et al*, 1973). Moreover, the fact that autoantibodies to other soluble monomeric proteins present in high concentrations, such as transferrin and complement components, have not been found in autoimmune disease states may be construed as evidence for their nonexistence (Nossal, 1983). Inasmuch as the clonal abortion/deletion theory requires that the epitope-mIg receptor interaction be of a certain threshold avidity to effect deletion, the existence, in normal animals, of B cells specific for autoantigens present in very low concentrations, such as thyroglobulin (Bankhurst *et al*, 1973; Roberts, *et al*, 1973; reviewed in Elson *et al*, 1977), growth hormone (Unanue, 1971), myelin basic protein (Yung *et al*, 1973) and nucleic acids (Bankhurst and Williams, 1975), does not seem to militate against it. Nevertheless, the role of clonal abortion/deletion in purging the B cell repertoire of reactivity to autologous serum components present in high concentrations remains to be determined.

Receptor Blockade

Antigen persisting on the B cell surface can cause a form of unresponsivness in mature B cells which has been termed receptor or effector cell blockade. Such a mechanism has been invoked to explain self tolerance at the B cell level, particularly to high concentrations of circulating antigens. Support for this notion comes from observations that antigen can inhibit antibody secretion from primed spleen cells (Schrader and Nossal, 1974; Klaus, 1976; Abbas and Klaus, 1977). If B cell unresponsiveness were solely the result of simple blockade by antigen, then removal of antigen, either enzymatically or by removal to an antigen-free environment, should restore responsiveness. Accordingly, proteolytic removal of antigen (Andersson *et al*, 1974) or incubation in tolerogen-free medium prior to challenge (Gronowicz and Coutinho, 1975; Aldo-Benson and Borel, 1976) reversed tolerance. Alternatively, triggering the B cell by a non-Ig dependent mechanism, such as with a mitogenic concentration of LPS, also restored responsiveness (Möller *et al*, 1976a). Other studies revealed, however, that B cell tolerance due to persistant antigen was not always reversible by such means. Thus, HGG tolerized (Elson and Taylor, 1977) or poly-D-(G,L) tolerized (Katz *et al*, 1972) B cells were not rendered responsive by enzymatic removal of antigen. Furthermore, tolerance induced by a short (< 16 hr) exposure to tolerogen was reversible, whereas that induced by prolonged exposure to tolerogen was not (Diener and Feldmann, 1972a). Even when the so-called "frozen" membrane was released by colchicine treatment, and new receptors appeared, the B cells remained unresponsive (Diener *et al*, 1976; Boyd and Schrader, 1982).

The distinction between reversible and irreversible blockade may depend upon the carrier, since tolerogenic concentrations of dextran irreversibly tolerized only those B cells also capable of responding to dextran as a PBA (Fernandez *et al*, 1979). Responsiveness in dextran specific B cells inducible by LPS was restored either by culture in dextran-free medium prior to challenge, or by removal of surface persisting dextran by dextranase. Taken together, these results suggest that receptor blockade occurs mainly with unusual antigens which persist on the cell surface (Aldo-Benson and Borel, 1974; Ault and Unanue, 1974; Ault *et al*, 1974; Nossal and Layton, 1976; Scott *et al*, 1979) and thus, is a carrier related phenomenon which may not reflect mechanisms involved in true self tolerance.

T Cell Mediated Unresponsiveness

T cell mediated B cell unresponsiveness can be the result of a negative T cell influence, or the absence of a required positive T cell influence in the case of TD antigens. The latter example pertains to *Ir* gene regulation of T_h cells. Although *Ir* genes can affect antibody responses (Pierce *et al*, 1976; reviewed in Benacerraf, 1981), this is the result of either a defective M0-T_h interaction (reviewed in Rosenthal, 1978; Schwartz *et al*, 1978), or a deficient T_h repertoire (Nagy *et al*, 1981). Thus, *Ir* genes only secondarily affect B cell responsiveness to TD antigens, and will not be further discussed here.

T cells having a negative influence on antibody responses, so-called T suppressor (T_s) cells, were first described by Gershon and Kondo (1970, 1971) and McCullagh (1970). Since then, the regulatory influences of T_s or their soluble products (Tada *et al*, 1975; Taussig, 1980) have been demonstrated in all types of immune responses (reviewed in Green *et al*, 1983; Dorf and Benacerraf, 1984). Some T_s are regulated by *Ir* genes (reviewed in Benacerraf *et al*, 1975), but this has only been demonstrated for a very limited number of antigens. T_s can be antigen specific (reviewed in Basten *et al*, 1975; Nachtigal *et al*, 1975; Tada *et al*, 1975), idiotype specific (reviewed in Green *et al*, 1983; Dorf and Benacerraf, 1984), allotype specific (reviewed in Herzenberg, 1983), epitope specific (reviewed in Herzenberg *et al*, 1983) or nonspecific (Rich and Pierce, 1973; reviewed in Dutton, 1975b; Pierce and Kapp, 1976b). Many antigens which induce B cell tolerance also induce T_s (Kölsch *et al*, 1975; Weigle *et al*, 1975). Whereas some investigators ascribe B cell unresponsiveness solely to T_s (Basten *et al*, 1975; Benjamin, 1975; Loblay *et al*, 1983) others demonstrate B cell tolerance in the absence of T_s (Elson, 1977; Parks *et al*, 1978; Metcalf *et al*, 1979; Waters *et al*, 1979). Alternatively, T_s may be present but nonessential in maintaining the tolerant state (reviewed by Parks and Weigle, 1980). Furthermore, the mechanism of maintaining unresponsiveness to a single antigen can change with time (Waters *et al*, 1979). In mice exposed to HGG *in utero*, B cell tolerance initially appeared to be maintained by a deletional mechanism; however, T_s were demonstrable at about 12 weeks after birth. Complicating matters further is the existence of memory T_s, which are only identifiable upon adoptive transfer and can be generated in the apparent absence of primary T_s induction (Loblay *et al*, 1983).

A major problem exists in deriving models for self tolerance based on the generation of T_s to extrinsic antigens in adult animals by deliberate immunization regimens. It is not at all clear whether self components are present *in vivo* under conditions conducive to T_s generation. There is, however, some evidence to suggest that T_s could be involved in maintaining tolerance to some self components. For example, *in vivo* depletion of T cells greatly increased the production of erythrocyte specific autoantibodies (Cunningham, 1975, 1976) suggesting that these self reactive B cells were normally under active suppression by T cells. The presence of T_s abnormalities in a wide variety of autoimmune diseases (Smith and Steinberg, 1983; reviewed in Flood, 1985) provides circumstantial evidence for the importance of T_s in preventing autoimmunity. However, it must be noted that the majority of such autoimmune diseases appear to have multiple cellular defects, making it difficult to ascribe the breakdown in self tolerance to a single abnormality.

In summary, while the importance of T_s in the normal homeostatic regulation of the antibody response has been clearly demonstrated, their role in the establishment and maintenance of self tolerance remains unclear. Most likely, some self antigens favor the induction of T_s, whereas others favor the functional or physical deletion of autoreactive B cells (Allison *et al*, 1971). The deletional and active suppression modes of tolerance may overlap, given the recent description of a T cell with both suppressive and cytotoxic properties (Heuer *et al*, 1982).

Experimental Tolerance to Authentic Self Antigens

Despite the extensive literature on tolerance to extrinsic antigens as models for self tolerance, there is surprisingly little indication that such an extrapolation is valid. Largely due to practical difficulties, remarkably few experiments have addressed the establishment and maintenence of self tolerance to authentic self antigens, at either the B cell or T cell levels. A classic early study by Triplett (1962), utilizing a novel experimental approach, established that immunological tolerance (presumably at the T cell level) to organ specific self antigens is acquired early in ontogeny, and is not inherited in the germline. Hypophysis primordia were removed from tree frog (*Hyla regilla*) embryos and "parked" in larval stage hosts until the hypophysectomized animals reached the metamorphic stage. Thus, the organ and immune system were allowed to differentiate in physically separated environments. At metamorphosis, the extirpated organ was reimplanted in the original host. Since hypophysectomized frogs are albino, due to the lack of hypophysis derived melanophore stimulating hormone, graft acceptance could be monitored by the return of the animal's natural dark color. The majority of grafts were rejected after this manipulation. However, if half of the organ had been left behind, the "parked" half was accepted upon return to the original host. This important control experiment verified that rejection in the first instance was not due to acquisition of foreign antigens by the "parked" organ. Recently, Rollins-Smith and Cohen (1983) have challenged this conclusion based upon their inability to repeat Triplett's findings. However, both the system and experimental protocol employed (including the frog genus and test organ used) were substantially different from Triplett's. Thus, it is difficult to ascertain whether the discrepancy lies in species specific differences in immunologic tolerance or in protocol differences.

Reichlein's (1972) early studies on the fine specificity of rabbit anti-human hemoglobin antibodies were interpreted in support of a deletional mechanism of B cell tolerance. Immunization of rabbits with various human hemoglobin mutants resulted in anti-hemoglobin antibodies specific only for those portions of the molecule not represented in rabbit hemoglobin. Thus, the presence of foreign and self determinants on the same molecule did not break tolerance at the B cell level, as has been reported for some antigens (Weigle, 1962; Cinader *et al*, 1967), presumably because such self reactive B cells were not present. However, anti-self hemoglobin antibodies were generated when the immunogen was the beta chain of human hemoglobin, suggesting, perhaps, that tolerance exists only to molecules in their native configuration.

More recent studies involving the B cell repertoire to autologous cytochrome c molecules also appeared to support a deletional mechanism for self tolerance. Jemmerson and

Margoliash (1979) examined the fine specificity of rabbit anti-rabbit cytochrome c antibodies, elicited upon immunization with polymerized rabbit cytochrome c. Such immunization had been previously shown to generate anti-rabbit cytochrome c antibodies (Reichlein *et al*, 1970). Although anti-self antibodies were generated, the magnitude of the response was considerably lower than that obtained with the heterologous cytochromes c, supporting the existence of a certain degree of self tolerance. The determinants recognized by such autoantibodies corresponded to the areas of the molecule which exhibited cross-species polymorphism. Interestingly, no autoantibodies were found that were specific for a region of the molecule which is conserved among all species tested. The authors speculated that autoimmunity to this region could not be risked. Thus, selective pressures may have eliminated such anti-self specificities from the germline repertoire.

In collaboration with Klinman's group, the cellular basis of the apparent self tolerance to the majority of autologous cytochrome c determinants was investigated using the splenic focus assay (Jemmerson *et al*, 1982). Allowing mIg$^-$ precursors to develop in the presence of BGG coupled cytochrome c and BGG specific help (Signals 1+2) prevented repertoire purging of anti-self specificities. B cells maturing in this environment contained considerably more anti-autologous cytochrome c specificities than already mature B cells subjected to the same environment.

These provocative studies suggest two different means of establishing tolerance to self antigens, one evolutionary and therefore inherited, and the other developmental, and therefore acquired.

Contrary to the conclusions supported by the above studies, there are reports of natural tolerance to self-antigens being maintained only at the T cell level, with no evidence for physical or functional deletion of B cells. Studies by Borel's group (Harris *et al*, 1982), perhaps more physiological than the Jemmerson studies, examined the status of T and B cells to an autologous serum protein (the fifth complement component, C5) without deliberately immunizing against it. Two congenic strains of mice were used, one genetically deficient in C5, and the other, normal. When cells from the C5 deficient strain were adoptively transferred to the normal strain, anti-C5 antibodies were readily detectable. Conversely, cells from normal animals were tolerant to C5 when challenged in C5 deficient hosts. It was further determined that only the T cells from the normal animals were tolerant, since normal B cells formed anti-C5 antibodies in the presence of nontolerant T cells from the C5 deficient strain. Nossal (1983) has criticized these studies on the basis of the large numbers of cells adoptively transferred and the relatively low dose of irradiation used on the adoptive hosts. However, as Nossal says, "taken at face value, the results provide another example of a monovalent antigen not leading to B cell tolerance" (Nossal, 1983).

The F cytoplasmic liver protein (MW 40,000) also fails to provide evidence for self tolerance at the B cell level. The F protein is polymorphic, and immunization of an F^1 strain with F^2 often results in the production of autoantibody (Iverson and Lindenmann, 1972). This is possibly an example of a B cell specific for self determinants being triggerred by a T cell specific for foreign determinants on the same molecule. Clonal abortionists/deletionists may argue that the molecule is present in serum concentrations too low to allow for an epitope-receptor interaction of sufficient avidity to tolerize F specific B cells (Nossal, 1983). Furthermore, clonal abortion/deletion may be operative at the T cell level, since T cells proliferated to syngeneic splenic adherent cells pulsed with the allogeneic but not the syngeneic form of the antigen (Sunshine *et al*, 1982).

The above discussion provides evidence both for and against deletional mechanisms as a means of purging the B cell repertoire of anti-self reactivities. Many more experimental studies with authentic self antigens must be performed before definite conclusions can be made. However, since most self antigens are monovalent and present in very low serum concentrations, deletion would not seem favoured as the major mechanism for effecting B cell tolerance. Inasmuch as the antibody repertoire is highly degenerate, widespread purging of anti-self reactivities would concomitantly decrease the available repertoire for foreign antigens (Nossal, 1983). Moreover, the extreme heterogeneity of self antigens with respect to size, valency, concentration, and anatomical location, would seem to necessitiate multiple strategies and failsafe mechanisms for the establishment and maintenance of self tolerance. That one all-encompassing mechanism protects us from *horror autotoxicus* is now regarded as naive and simplistic, especially in view of the myriad of interacting cell types and lymphokines involved in immune induction and the complex immunoregulatory systems which have been described.

D. Accessory Cells in B Cell Tolerance

Insofar as deletional theories of B cell tolerance propose that the direct interaction of antigen and B cells is tolerogenic, Mitchell and Nossal's observation (1964) that the RES of young animals, in contrast to their adult counterparts, failed to sequester ^{125}I-POL in lymphoid follicles, would appear to ascribe a pivotal role in tolerance induction to the RES. That A cells from young animals are functionally immature was confirmed by Karthigasu *et al* (1965) and Landahl (1976). Clonal deletion/abortion could, therefore, be a consequence of the inherent sensitivity of immature B cells to negative signalling, deficient A cell function facilitating direct antigen-B cell encounters, or a combination of both. A cell immaturity alone (Landahl, 1976) or in conjunction with high T_s activity (Argyris, 1984) have thus been suggested as the cause of both the poor immunologic responsiveness and susceptibility to tolerance induction observed in young animals.

Influence of the RES on tolerance induction in adult animals has also been reported. Studies by Frei *et al* (1965) indicated that "biofiltration" of BGG through animals removed only the immunogenic material; antigen recovered from the serum (ie., antigen which had not been sequestered in lymphoid organs by the RES) was shown to be highly tolerogenic upon transfer to naive recipients. The authors speculated that antigen must be phagocytosed to be immunogenic; that which is not susceptible to phagocytosis would be tolerogenic. A similar suggestion was made by Glynn and Holborow (1965). In support of this concept, Mosier (1975, 1976) found that A cell presented DNP-D-(G,L) was nontolerogenic compared to the soluble form, and Pillai and Scott (1981) observed that the addition of MØ abrogated tolerogen mediated growth inhibition of B cell colonies. Nachtigal *et al* (1968) suggested that the delayed restoration of A cell function rendered 12 week post-irradiation animals susceptible to tolerance induction. Thus, antigen pulsed A cells restored immunocompetence in irradiated animals

(Gallily and Feldman, 1966, 1967) which had undergone lymphoid repopulation, but were still tolerance susceptible (Nachtigal *et al*, 1968). At variance with the results of Nachtigal *et al* (1968) and Frei *et al* (1965), Howard and Siskind (1969) found persisting extracellular pneumococcal polysaccharide to be highly immunogenic. However, the determination of the balance between induction and tolerance by the RES may depend on both the particular properties of the antigen, and on the heterogeneity of A cells, since different A cell subsets have different functional capabilities (Lee, 1980; Guidos *et al*, 1984).

Humphrey demonstrated in 1981 that different patterns of antigen localization by the RES, likely dependent upon the physical properties of an antigen, were correlated with different functional consequences for the immune system. The tolerogenic properties and cellular localization patterns of a variety of TI natural and synthetic hapten-polysaccharide conjugates were determined. Interestingly, the tolerogenic conjugates were trapped predominantly in MØ of the splenic red pulp, whereas immunogenic conjugates were localized primarily in the marginal zone MØ of the splenic white pulp. Others have also provided evidence that antigens localized *in vivo* in red pulp MØ are poorly immunogenic (Joshua *et al*, 1980). Moreover, marginal zone MØ are situated in areas of B cell traffic and B cells adhere to marginal zone MØ *in vitro* (Humphrey, 1980), leading to the suggestion that antigen trapping by marginal zone MØ leads to immunity, whereas trapping by red pulp MØ does not. Tolerance may thus result from the direct interaction of B cells with persisting free antigen which has failed to become localized by stimulatory marginal zone MØ, and/or has been localized by nonstimulatory red pulp MØ. The exact mechanism of selective localization of polysaccharide antigens remains unknown, however the neutral polysaccharides being more immunogenic and the acidic polysaccharides tolerogenic. These results provide an example whereby differential handling of antigen by the RES, a carrier determined property, determines the balance between immunity and tolerance in hapten specific B cells.

An extensive series of studies by Leskowitiz and colleagues indicated that strain specific variations in susceptibility to BGG induced tolerance may also be the result of differential antigen handling by the RES. A similar conclusion was reached by Fujwara and Cinader

(1974) from their study on resistance to rabbit gamma globulin induced tolerance in SJL mice. Golub and Weigle (1969) first noted that, in contrast to DBA/2 mice, BALB/c mice were relatively resistant to BGG induced tolerance. However, Lukic and Leskowitz (1974) demonstrated that carrageenan, which is preferentially cytotoxic for MØ, destroyed this resistance in BALB/c mice. Moreover, radiation chimeras made between the two strains behaved like the host with respect to tolerance sensitivity (Das and Leskowitz, 1974). These conclusions were extended by Lukic *et al* (1975) and Cowing *et al* (1977), who showed that sensitivity to BGG induced tolerance could be decreased by MØ activation *in vivo*, and that BALB/c, but not DBA/2, SAC could render BGG capable of inducing tolerance in BALB/c mice. This was an *in vitro* biofiltration technique analgous to the *in vivo* biofiltration studies of Frei *et al* (1965). Cowing *et al* (1979) ultimately ascribed this strain specific difference in MØ function to the presence, on BALB/c MØ only, of Fc receptors for the IgG$_3$ subclass of HGG, which displayed strain specific tolerogenic properties similar to BGG. Thus, the relative inefficiency of DBA/2 MØ in taking up heterologous gamma globulins supports the model of Frei *et al* (1965) purporting that antigens not susceptible to phagocytosis are tolerogenic. However, in view of the fact that this effect appears to be Fc mediated, the general applicability of this model of tolerance must be considered cautiously.

Contrary to the Leskowitz *et al* phenomenon just described, Phipps and Scott (1983) have more recently reported that tolerance induction to FLU-sheep gamma globulin (SGG) conjugates was 10 times more effective in the presence of tolerogen pulsed A cells. The protocol involved preculturing spleen cells with normal or tolerogen pulsed A cells for 24 hr prior to a 3 day culture with FLU-Ba or FLU-POL. A cell induced tolerance was T cell independent, not MHC restricted, and relatively hapten specific. A cell populations rich in Ia$^+$ cells could induce tolerance, as could LPS stimulated A cells. LPS stimulation presumably increased A cell secretion of IL-1. The fact that A cell induced tolerance was only observed with gamma globulin tolerogens makes this study subject to the same criticisms raised earlier. In fact, A cells abrogated the tolerogenic capacity of FLU-D(G,L), in agreement with previous results (Mosier, 1975, 1976).

A potentially interesting aspect of this work pertains to the differential effects obtained when various MØ-like and dendritic cell (DC)-like tumor lines were assessed for their tolerogenic capacity. In contrast to peritoneal A cells, P388AD.2, a DC-like line (Cohen and Kaplan, 1981) enhanced, rather than diminished, the FLU specific AFC response. This enhancement was mediated by an MHC restricted interaction between P388AD.2 and Thy-1^+, Lyt-2^+ T cells which exerted their effect only during the preculture phase of the experiment, since subsequent T cell depletion had no effect (Phipps *et al*, 1984). Examination of a number of cloned MØ-like and DC-like tumor lines for this enhancing activity showed that only Ia^+ clones were functional, and Fc receptor expression was not a requirement. The authors felt that this mitigated against Fc receptor mediated uptake of antigen as being responsible for their results. However, this can only be said of the enhancement phenomenon. In terms of tolerance induction, only FcR^+ cells (peritoneal MØ and J774.5R) were capable of presenting FLU-SGG in a tolerogenic fashion (Phipps and Scott, 1983; Phipps *et al*, 1984). Thus, while the significance and the mechanism of the enhancement phenomenon remain unclear, the greater efficiency of tolerance induction in the presence of FcR^+ A cells could simply be due to presentation of a locally high concentration of antigen in a multivalent array, thus favoring tolerance over immunity (Feldmann, 1972b).

Diener *et al* (1976) have reported that A cells can determine the balance between tolerance and immunity to a non-gamma globulin tolerogen. Tolerance induced to high concentations of POL *in vitro* was "broken" if A cells were added for 12 hours prior to immunogenic challenge. Only A cells which had been unpulsed, or pulsed with immunogenic concentrations of POL, could break tolerance. A cells incubated with a tolerogenic dose of POL failed to break tolerance but still functioned normally in the *in vitro* AFC response to sheep red blood cells (SRBC). Thus, the A cells were not generally compromised. The apparent specificity of A cells in this system is difficult to explain. Taken at face value, the results appear to indicate that A cells pulsed with tolerogenic concentrations of POL somehow fail to interact with tolerogen pulsed B cells in an effective manner. A direct A cell-B cell interaction in the response to POL, while not proven, is at least consistent with the demonstrated T cell independence (Diener *et al*, 1971) and A cell dependence (Lee *et al*, 1976) of this response. These results will be further considered in the light of data to be presented in this thesis, which also bear on the antigen specific participation of A cells in TI B cell tolerance.

Another example of antigen specific failure of A cells from tolerant mice was recently reported by Biasi *et al* (1983). In this system, A cells from mice neonatally tolerized to Moloney murine leukemia virus were selectively defective in promoting *in vitro* generation of virus specific, but not allo specific, cytotoxic T lymphocytes in A cell deficient spleen cells. After neonatal virus infection, the A cell progenitors became productively infected and proviral DNA was inherited by the progeny of infected A cells. Thus, virally encoded antigens could be detected on the A cell surface. The authors suggested that since the "foreign" viral determinants were encoded by viral DNA within the A cell DNA, they may not be processed in the same manner as an extrinsic foreign antigen. This raises the possibility that A cells may, under some circumstances, distinguish between self and nonself proteins, and process only the latter. Indeed, Raff (1982) suggested that this may be necessary to prevent the saturation of A cells by self molecules. However, such discriminatory capabilities have not yet been demonstrated for A cells. The idea that A cells require antigen specific lymphocyte products (eg. antibody or T cell factors) to recognize foreign antigens (Raff, 1982) is untenable; homogeneous A cell lines take up foreign antigens efficiently in the absence of lymphocytes. Furthermore, the apparent discrimination by A cells between self and foreign antigens reported by Biasi *et al* (1983) may be a special case since A cells did not distinguish between self and nonself forms of serum F glycoprotein (Czitrom *et al*, 1981).

A role for MØ or A cells has also been suggested in antibody or immune-complex mediated suppression. Pierce (1969b) reported that antibody did not directly suppress B cells; rather, it neutralized the antigen at the macrophage dependent phase of the response. Haughton and Adams (1970) reached a similar conclusion. Abrahams *et al* (1973) found that MØ were required for immune complex suppression, and Hutchinson and Zola (1978) proposed that immune complex inhibition *in vivo* may be the result of opsonization of B cells leading to their phagocytosis and destruction by MØ (reviewed in Hutchinson, 1980). Miranda *et al*

(1972) similarly postulated that B cells "blocked" with persisting antigen may be vulnerable to phagocytosis by MØ.

In addition to direct MØ-B cell interactions in the induction of tolerance, MØ have also been implicated as modulators of T_s mediated unresponsiveness. In the latter case, the preferential stimulation of T_s in genetic nonresponders to the synthetic antigen $Glu^{60}Ala^{30}Tyr^{10}$ (GAT) can be circumvented by immunization with MØ associated GAT, rather than soluble GAT (Kapp *et al*, 1975; reviewed in Pierce, 1980). Thus, the mode of antigen presentation to T cells (soluble *vs* MØ associated) may be a major factor in controlling the balance betwen T_h and T_s.

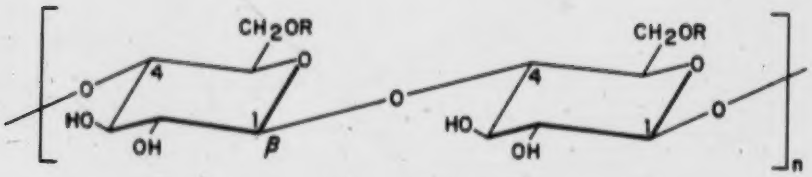
The function of MØ in mediating nonspecific T cell suppression is well characterized. Such suppression is effected by soluble immune response suppressor factor (SIRS) produced by Con A or interferon activated murine Ly-2^+ T cells (Aune and Pierce, 1981a; Aune *et al*, 1983). At least two molecular species of this factor exist - SIRS-α and SIRS-β, having molecular weights of 14,000 and 21,000 daltons respectively (Aune *et al*, 1983). Consistent with this, two separate poly-A^+ mRNA species were identified which were translated *in vitro* into two forms of SIRS (Nowowiejski-Wieder *et al*, 1984). More recently, additional isoforms of SIRS-α have been identified (Webb *et al*, 1985). The biological activity of SIRS is dependent upon MØ (Tadakuma and Pierce, 1976) to convert it to an active form ($SIRS_{ox}$) (Aune and Pierce, 1981a,b), possibly by an H_2O_2 dependent mechanism, since H_2O_2 treated SIRS is also biologically active (Aune and Pierce, 1981c). $SIRS_{ox}$ inhibits mitogen induced proliferation in B and T cells, *in vitro* AFC responses, and the division of tumor cells (Aune and Pierce, 1981b). The inhibitory effects of $SIRS_{ox}$ can be reversed by reducing agents, suggesting that it may be an oxidizing agent (Aune and Pierce, 1981b,c). Recent studies have revealed that $SIRS_{ox}$ inhibits microtubule functions necessary for cell division (Irons *et al*, 1984). However, growth factors, such as IL-1, IL-2 and epidermal growth factor, reversed this inhibition (Aune, 1985).

II. Development of the Research Program

Owing to the difficulty of inducing experimental tolerance to extrinsic antigens in adult animals, immunologists have relied heavily on particular tolerogens selected for their efficiency in this regard. While this approach has led to the formulation of several theories of self-nonself discrimination, the relevance of unresponsiveness to model tolerogens as a reflection of natural mechanisms of self tolerance has never been firmly established. For example, the voluminous literature supporting clonal deletion/abortion as the mechanism of B cell self-nonself discrimination is based almost entirely on experiments using gamma globulin carriers to induce hapten specific tolerance. However, the Fc dependence of gamma globulin induced tolerance is difficult to reconcile with the notion that only epitope-Ig receptor interactions are germane in tolerance by clonal deletion/abortion. Moreover, the existence of Fc dependent negative feedback regulation of immunoglobulin production by interactions with B cell Fc receptors considerably complicates interpretations of experiments utilizing gamma globulins as model tolerogens.

Because the general applicability of tolerance by gamma globulin induced clonal deletion/abortion is limited, for many years Diener's laboratory has used several different tolerogens to uncover alternate mechanisms of B cell unresponsiveness. Such investigation has illuminated the importance of the carrier molecule in determining the mechanism of tolerance. In 1979, two completely nonimmunogenic carriers were shown to be capable of inducing profound B cell tolerance. Carboxymethyl cellulose (CMC, MW 200,000) and methyl cellulose (MC, MW 86,000) are polymeric carbohydrates consisting of glucopyranoside rings joined in β 1:4 glycosidic linkages (Figure 1). The tolerance induced by hapten-CMC/MC conjugates was shown to be hapten specific, long lasting, and T cell independent, yet carrier dependent (Diner *et al*, 1979; Diener *et al*, 1981; von Borstel *et al*, 1983). Thus, the CMC/MC mediated unresponsiveness appeared to be effected by a novel mechanism.

Based on the observations that 1) A cells determine the balance between immunity and tolerance to POL (Diener *et al*, 1976), and, 2) CMC/MC tolerance is carrier dependent, T cell


CH2OR
O
4
1
β
HO
OH
O
CH2OR
O
4
1
HO
OH
O
n

cellulose R = H
methyl cellulose R = CH_2
carboxymethyl cellulose R = CH_2COOH

Figure 1. Chemical structures of CMC and MC tolerogens.

independent (as shown by studies using T cell deficient *nu/nu* and adult thymectomized, irradiated, fetal liver reconstituted mice, and cellular mixing experiments), the studies initially undertaken in this thesis were designed to investigate the possible role of adherent accessory (A) cells in B cell tolerance induced by hapten-CMC/MC conjugates. To avoid the potentially complicating influences of A cell-T cell interactions, an A cell dependent, T cell independent (TI) system of *in vitro* B cell triggering was employed (Figure 2).

Studies presented in *Chapter IV* revealed that A cells from hapten-CMC/MC tolerant mice are hapten specifically deficient in reconstituting immunocompetence in A cell deficient spleen cells. Moreover, the induction of tolerance by hapten-MC *in vitro* was found to be critically dependent upon the presence of A cells. In order to investigate possible mechanisms of A cell participation in the induction of hapten specific unresponsiveness, the precise functions of A cells in our TI B cell activation system were assessed. Therefore, studies designed to elucidate the exact nature of A cell participation in TI B cell activation form the basis of *Chapter V*.

The cellular and molecular basis of A cell function in T cell activation is relatively well understood. In response to most antigens, T cells recognize processed antigen in association with MHC molecules on the surface of A cells. Furthermore, A cell derived IL-1 has been shown to provide a necessary activation signal to T cells in some systems. There have been suggestions that A cells mediate identical functions in T cell activation and TI B cell activation, due to the dependence of the latter on nonspecific T cell derived factors. However, the studies presented in *Chapter V* suggest that IL-1 secretion by A cells is their only essential function in TI B cell activation. The interpretation of these studies was greatly facilitated by two recent technological advances. DSI serum-free culture medium, developed in Dr. Diener's laboratory, and pure recombinant IL-1, kindly provided by Dr. Peter Lomedico (Hoffman LaRoche) allowed A cell function in TI B cell responses to be unambiguously ascribed to IL-1 secretion. This important finding enabled us to begin the investigation of mechanisms by which A cells mediate hapten specific B cell unresponsiveness. These studies form the basis of *Chapter VI*.

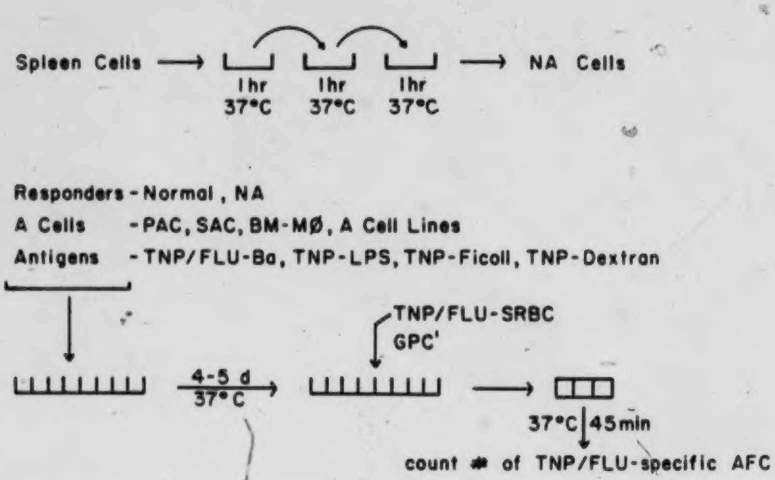

Spleen Cells
1hr 37°C
1hr 37°C
1hr 37°C
NA Cells
Responders - Normal, NA
A Cells - PAC, SAC, BM-MØ, A Cell Lines
Antigens - TNP/FLU-Ba, TNP-LPS, TNP-Ficoll, TNP-Dextran
4-5 d
37°C
TNP/FLU-SRBC
GPC'
37°C
45min
count # of TNP/FLU-specific AFC

Figure 2. Protocol for *in vitro* stimulation of A cell dependent AFC responses by TI antigens.

III. Materials and Methods

The protocols described in this chapter were used throughout this thesis. Methods which were used only in a particular study will be described in the *Materials and Methods* section of the relevant chapter.

Mice. CBA/CaJ (H-2^k), DBA/2 (H-2^d), and C57BL/10 (H-2^b) mice, 8 to 12 wk old were obtained from the Ellerslie Small Animal Farm, University of Alberta, Edmonton, Alta.

Antigens. Brucella abortus (Ba) organisms were obtained from National Veterinary Service Lab., IO. Trinitrophenyl (TNP) conjugates of Ba were prepared as follows. Forty mg of trinitrobenzene sulfonic acid (TNBS, Sigma Chemical Co., St. Louis, MO) were dissolved in 4 ml of 4% $NaHCO^-_3$, pH 9.5, and added at once to 4 ml of a suspension of 1.4 x 10^{11} bacteria/ml in saline. After 1 hr, the organisms were washed 3 times and resuspended in 4 ml of sterile saline. Conjugates of FLU-Ba were prepared in the following manner. Twenty mg of fluorescein isothiocyanate (FITC) (isomer 1, Sigma Chemical Co., St. Louis, MO) were dissolved in 6 ml of 0.05 M HCO^-_3, pH 9.5, and the solution filtered through a 0.22 μm Millipore filter (Millipore Corp., Bedford, MA). Four ml of a suspension of 1.4 x 10^{11} bacteria/ml were added at once to the FLU solution and the resulting mixture was allowed to react at room temperature for 1 hr. The organisms were then washed 3 times and resuspended in 4 ml of sterile saline.

To prepare TNP-LPS, 30 mg *Salmonella typhosa* 0901 LPS (Difco Laboratories, Detroit, MI) dissolved in 5 ml 0.1 M Na_2CO_3 (pH 9.0) were admixed with 30 mg TNBS dissolved in 3 ml of water. The reaction was allowed to proceed overnight at 4° C, after which the solution was dialyzed extensively against water. The conjugation ratio was determined by dry weight determination and the absorption at 360 nm.

The preparation of TNP-ficoll and TNP-dextran has been described elsewhere (Diner *et al*, 1979).

Spleen Cell Suspensions. Mice were killed by cervical dislocation and their spleens removed into phosphate buffered saline (PBS) supplemented with 10% volume/volume (v/v) fetal calf serum (FCS) ("Rehatuin", Reheis Chemical Co., Phoenix, AZ). Spleen cell suspensions were obtained by gently squeezing the tissue between tweezers in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 5% FCS under sterile conditions. Debris was removed by sedimentation at 1 *g* for 10 min after which the cells were centrifuged at 400 *g* for 7.5 min and resuspended in RPMI containing 10% FCS and 5 x 10^{-5} M 2-ME, or DSI serum free medium (Quadralogic Co., Vancouver, B.C.) where indicated.

Depletion of Adherent Accessory (A) Cells. Fifty million spleen cells in 5 ml of RPMI supplemented with 10% FCS and 5 x 10^{-5} M 2-ME were fractionated into adherent and nonadherent (NA) subpopulations by incubation for 1 hr in Falcon 3003 tissue culture dishes at 37° C in a 10% CO_2/air atmosphere. NA spleen cells were transferred to a new dish and the procedure was repeated twice, whereupon the NA cells were washed, resuspended in medium, and counted.

Peritoneal Accessory Cells (PAC). Cells were harvested from the peritoneal cavity of normal or tolerant mice according to the technique of van Furth and Cohn (1968). Mice were killed by gentle cervical dislocation to avoid contamination of the peritoneal cavity with blood. After peeling back the fur to expose the abdomen, 3 ml of PBS containing 5% FCS were injected into the peritoneal cavity using a syringe with a 26 gauge x 1/2 in needle. Following gentle massage of the abdomen to ensure adequate lavage of the peritoneum, the fluid was removed by inserting a Pasteur pipette into a tiny hole cut into the peritoneal wall. Cells were centrifuged at 450 *g* for 7 min at 4° C and resuspended in 5 ml PBS containing 5% FCS prior to irradiation.

Splenic Accessory Cells (SAC). Splenic A cells were obtained after the first incubation of the *Depletion of Adherent Accessory Cells* procedure. Adherent cells were harvested from the plate using a rubber policeman, after which they were washed and resuspended in PBS containing 5% FCS prior to irradiation.

Irradiation of Accessory Cells. All accessory cells were γ-irradiated with 1500 rad in a 'Gamma Cell 40' ^{137}Cs source (Atomic Energy of Canada Limited, Ottawa, Ont.). For irradiation, cells were suspended in PBS or RPMI with 5% FCS without 2-ME. Following irradiation, cells were centrifuged at 450 *g* for 7 min at 4° C and resuspended in the appropriate medium for tissue culture.

Cell Counts. The modified Neubauer hemocytometer (American Optical, Inc., Buffalo, NY) was used for all cell counts. Viability was determined by the eosin dye exclusion test (Hanks and Wallace, 1958).

In Vitro Antibody Forming Cell Responses. One million viable unfractionated or NA spleen cells from normal or tolerant mice were cultured with the various TI antigens at the indicated concentrations in flat-bottomed, 96 well Linbro tissue culture plates (Flow Laboratories, Toronto, Ont.) in a volume of 0.3 ml medium. Tissue culture medium was either RPMI containing 10% FCS and 5 x 10^{-5} 2-ME or DSI serum-free medium as indicated in each chapter. Cultures were incubated for 4 days at 37° C in a 10% CO_2/air atmosphere. To prepare cultures for enumeration of AFC, plates were centrifuged at 450 *g* for 7 min at 4° C. The medium was replaced with 0.2 ml Mishell-Dutton balanced salt solution (MDBSS) (Mishell and Dutton, 1967), whereupon the centrifugation step was repeated. The cells were then resuspended in 0.1 ml MDBSS prior to the antibody forming cell assay.

Antibody Forming Cell Assay Following In Vitro Challenge. The number of direct IgM AFC were determined by the Jerne plaque assay (Jerne and Nordin, 1963) as modified by Cunningham and Szenberg (1968), using TNP or FLU conjugated SRBC as targets. Briefly, 0.05 ml of haptenated SRBC targets (10% v/v) plus 0.05 ml of guinea pig complement (C') (Flow Laboratories, Inglewood, CA) diluted 10 times in MDBSS were added to each well containing spleen cells in 0.1 ml MDBSS, as described above. After thorough mixing, 0.05 ml of the mixture was transferred into double-walled slide chambers which were then sealed with wax. Slides were incubated at 37° C for 45-60 min prior to counting plaques under a stereoscopic dissecting microscope (American Optical, Inc., Buffalo, NY). Results are expressed as the mean ± standard deviation (SD) of quintuplicate cultures per 10^6 spleen cells. The numbers of AFC in cultures without antigens were always less than 20, and have been subtracted from all experimental groups.

Haptenation of SRBC. SRBC in Alsever's solution were purchased from Morse Biological Supplies, Edmonton, Alta., and stored at 4° C for no longer than 2 wk. Cells were washed 3 times in saline containing 1% Ca^{2+} and 1% Mg^{2+} prior to haptenation.

To prepare TNP-SRBC (Hudson and Hay, 1976), 1.0 ml packed, washed SRBC was added to 30 mg TNBS dissolved in 10 ml cacodylate buffer (pH 9.5). After 10-12 min at room temperature, 30 mg glycylglycine (Sigma Chemical Co., St. Louis, MO) dissolved in 35 ml PBS containing 1% Ca^{2+} and 1% Mg^{2+}, were mixed with the SRBC solution. After 5 min at room temperature (in the dark), the cells were washed extensively (4-5 times) with saline, until the supernatant was completely clear. After the last wash, the cells were suspended in MDBSS such that the final concentration of SRBC was 10% v/v.

To prepare FLU-SRBC (Scott, 1980), 10 mg FITC were dissolved in 5 ml of 0.05 M bicarbonate buffer (pH 9.5). The slightly turbid solution was filtered through a millipore filter (diamter 0.22 μm) and mixed with 0.5 ml packed, washed SRBC. After 30 min at room temperature (in the dark), 40 ml of PBS were added and the cells were washed 4-5 times, until the supernatant was completely clear. The cells were suspended in MDBSS after the last wash, to a final concentration of 10% SRBC v/v.

IV. Antigen Specific Failure of Accessory Cells From Tolerant Mice to Reconstitute Immunocompetence in Accessory Cell Deficient Spleen Cells

A. Introduction

B cell tolerance is commonly defined as the functional deletion of immunocompetent cells by the direct binding of an antigen at a sufficiently high concentration and with sufficient affinity to surface Ig receptors. In disagreement with this notion are observations which indicate that the control of tolerance, be it induced by clonal deletion or maintained by active suppression, is largely determined by the molecular nature of the antigen and less so by epitope affinities or degrees of immunogenicity (Diner *et al*, 1979; Waters and Diener, 1983). For example, hapten specific tolerance may be induced in neonatal mice with hapten derivatives of HGG or BSA, yet clonal analysis revealed that deletion of immunocompetent B cells is induced by hapten derivatized HGG only. Unresponsiveness in mice treated from birth with hapten derivatized BSA was shown to be due to carrier specific suppressor T cells in the presence of an undiminished clone size of hapten specific B cells (Waters and Diener, 1983). Hapten derivatives of CMC and MC, which induce profound hapten specific tolerance in both adult and neonatal mice, provide examples of carrier related, yet T suppressor cell independent tolerogens (Diner *et al*, 1979; von Borstel *et al*, 1983). Upon appropriate chemical alteration of the carriers, these conjugates are no longer capable of inducing tolerance regardless of the ontogenic stage of the animal, even though such alteration does not reduce the conjugates' binding avidity for either free hapten specific antibody or hapten binding B cells (von Borstel *et al*, 1983).

The present study is concerned with the analysis at the cellular level of the mechanisms by which hapten derivatized CMC or MC induce hapten specific tolerance. We report that tolerance to these nonimmunogenic conjugates is controlled by radioresistant A cells contained ~~in~~ the spleen or peritoneal fluid of normal mice. Restoration of immunocompetence in A cell depleted spleen cells is possible only with A cells from the peritoneal cavity or the spleen of nontolerant mice. Moreover, the inability of A cells from tolerant animals to restore immuncompetence was found to be specific only for the hapten to which tolerance was induced. This complements and extends earlier work from this laboratory which demonstrated for the first time the control by A cells of *in vitro* induced tolerance to POL (Diener *et al*, 1976, 1977).

B. Materials and Methods

Antigens. TNP-Ba and FLU-Ba were prepared as described in *Chapter III*. Cultures were challenged with 14 x 10^8 TNP-or FLU-Ba organisms.

Tolerogens. CMC (medium viscosity), MC (4000 centipoises), TNP-lysine, and cyanogen bromide (CNBr) were purchased from Sigma Chemical Co., St. Louis, MO).

To prepare TNP-CMC, 300 mg of CMC were transformed into the aminoethyl derivative as previously described (Diner *et al*, 1979). Twenty percent Na_2CO_3 in H_20 was added dropwise to 35 ml (4 mg/ml) of this derivative until the pH reached 9.5, after which 5 mg of TNBS or FITC in 5 ml H_20 were added at once. The pH was adjusted to 9.5, and the reaction mixture left overnight at room temperature. The intensely yellow solution was then dialyzed extensively against H_20 for 2 days. The ratio of TNP or FLU per 100,000 daltons of CMC was calculated by dry weight determination and the absorption at 360 nm and 495 nm, respectively..

To prepare TNP-MC, 300 mg MC were allowed to dissolve in 40 ml H_20 overnight, after which 100 mg CNBr in 10 ml Na_2CO_3 were added over 15 min with constant stirring. The pH of the solution was kept between 10.5 and 11.0 for 30 min by dropwise addition of 0.1 N NaOH. Na_2CO_3 was then added to the solution to a final concentration of 0.1 N. Then, 15 mg of TNP-lysine dissolved in 5 ml Na_2HCO_3 were added. The reaction mixture was stirred overnight at room temperature, then dialyzed extensively against H_20. The ratio of TNP per 100,000 daltons MC was calculated by dry weight determination and absorption at 360 nm.

In Vivo Tolerance Induction. Adult CBA/CaJ mice were injected intravenously with 350 μg/ml TNP-CMC or TNP-MC (3 mice/group). After 48 hr, spleens and or peritoneal cells were removed for culture.

Purification of Splenic MØ. The purification and characterization of splenic MØ was carried out as described by Guidos *et al* (1984), and is summarized in Figure 3. Briefly, splenic A cells were first enriched by irradiation and overnight adherence. Viable cells were recovered the next day by centrifugation over ficoll-hypaque (density 1.09 g/cm^3), after which a MØ enriched (FcR$^+$) fraction was obtained by rosetting for 1 hr at 4° C with IgG coated SRBC, followed by centrifugation through 56.05% percoll (density 1.08 g/cm^3). Routinely, the MØ fraction contained 98% FcR$^+$ cells.

Depletion of T Cells. Mice were injected 48 hr prior to use with 0.04 ml of anti-thymocyte serum (ATS, M.A. Bioproducts, Walkersville, Md.) diluted 1:5 in saline (Leibson *et al*, 1981). To deplete Thy-1 and Ly-2 positive cells, no more than 20 x 10^6 cells were suspended in 1 ml of Leibowitz medium containing 0.1% gelatin (Gibco Laboratories, Grand Island, N.Y.), 5 x 10^{-5} M 2-ME, and anti-Thy-1.2 and anti-Ly-2.1 antibodies (New England Nuclear, Boston, MA) at the appropriate titres. The cells were kept at 4° C for 90 min, centrifuged at 400 *g* for 7.5 min and resuspended in 1 ml of a selected batch of rabbit serum as a source of C' (Boyse *et al*, 1970) at a dilution of 1:5 for 45 min at 37° C. Cells were then washed 3 times before further treatment or culture. This treatment of cells from ATS treated mice routinely killed 80-90% of thymocytes from 4-6 wk old mice, 20-30% of unfractionated spleen cells and 30-40% of NA spleen cells.

C. Results

Failure of A Cells From Tolerant Mice to Restore Immunocompetence of A Cell Depleted Spleen Cells

Mice injected with the nonimmunogenic hapten derivatives of CMC or MC are hapten specifically tolerant upon challenge *in vivo* or *in vitro* with a hapten derivatized immunogenic, TD or TI carrier (Diner *et al*, 1979; von Borstel *et al*, 1983). This state of tolerance is not due to receptor blockade and is independent of T cells (Diner *et al*, 1979). In view of this observation and an earlier finding that tolerance to the polymeric form of the flagellar protein POL is under the control of radioresistant, Thy-1$^-$ A cells (Diener *et al*, 1976, 1977), we have

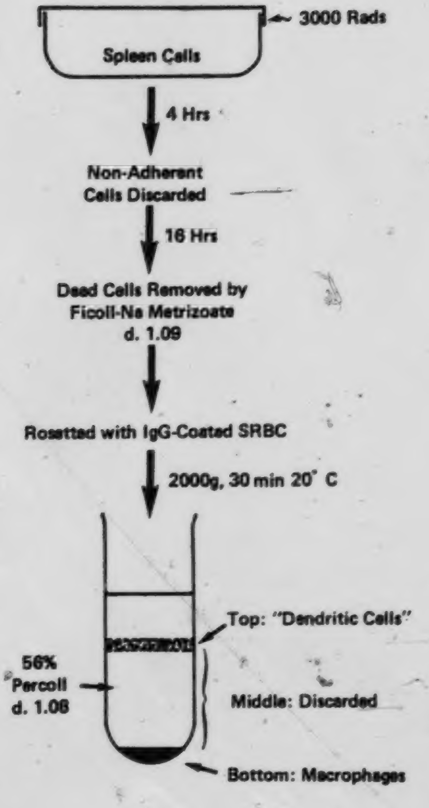

3000 Rads
Spleen Cells
4 Hrs
Non-Adherent Cells Discarded
16 Hrs
Dead Cells Removed by Ficoll-Na Metrizoate d. 1.09
Rosetted with IgG-Coated SRBC
2000g, 30 min 20° C
Top: "Dendritic Cells"
56% Percoll d. 1.08
Middle: Discarded
Bottom: Macrophages

Figure 3. Protocol for purification of splenic MØ.

analyzed the possibility that A cells may also be involved in hapten specific tolerance induced by hapten derivatized CMC or MC. NA spleen cells (10^6 cells/culture) were cultured with irradiated peritoneal A cells (PAC) from either normal mice or mice that had been rendered tolerant to TNP-CMC 48 hr previously. Only PAC from normal mice were capable of restoring immunocompetence to TNP-Ba (Figure 4). However, PAC from TNP tolerant mice were as effective as normal PAC in restoring immunocompetence to a different hapten, FLU (Figure 4). This raised the possibility that the state of unresponsiveness induced by the tolerogen may be due to the transient, hapten specific, A cell dependent suppression of otherwise normal B cells. However, immunocompetence to TNP in NA cells from tolerant mice could not be restored by normal PAC (Figure 4), indicating that B cells were indeed tolerant. Furthermore, A cells from tolerant mice were nonfunctional only for the hapten to which the mice had been tolerized (Figure 5), thus confirming the hapten specificity of the A cell defect.

For reasons of convenience we have used PAC rather than splenic A cells (SAC) to reconstitute NA spleen cell cultures. To confirm that in this system, SAC behave similarly to PAC, NA spleen cells were reconstituted with SAC from normal or tolerant mice (Figure 6). Both were functionally comparable. Thus, we are confident that experiments using PAC as the reconstituting elements are representative of events occuring in the spleen.

Induction of Hapten Specific A Cell Defect *In Vitro*

The preceeding experiments suggested that the inability of A cells from tolerant mice to mediate immunocompetence *in vitro* to the respective hapten may result from the direct interaction of these cells with the tolerogen. To assess this possibility, PAC from normal mice were irradiated, preincubated at 4° C for 90 min with TNP-MC and washed 3 times prior to culture with NA spleen cells in the presence of TNP-Ba or FLU-Ba. PAC which had been preincubated with TNP-MC failed to restore immunocompetence to TNP but remained unaffected in their capacity to restore the FLU specific response (Figure 7). Further, to ascertain whether PAC are required for the induction of B cell tolerance by TNP-MC, NA

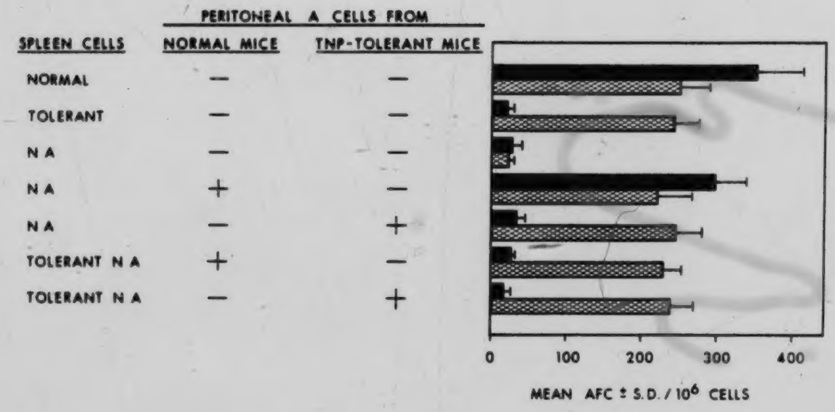


Figure 4. A cells from tolerant mice fail to reconstitute NA spleen cells. Mice were rendered tolerant by injection of 300 μg TNP$_6$MC i.v. 48 hr before removal of spleens or PAC for culture. 2 x 10^5 irradiated PAC from normal or tolerant mice were used to reconstitute NA spleen cells. Cultures were challenged with TNP-Ba (solid bars) or FLU-BA (hatched bars).

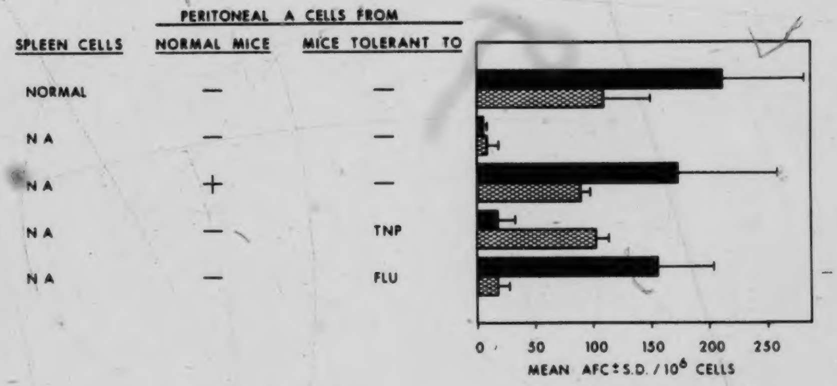


Figure 5. The failure of A cells from tolerant mice to reconstitute immunocompetence is hapten specific. A cells were obtained 48 hr after i.v. injection of 300 μg of the tolerogens TNP$_6$CMC or FLU$_5$CMC. 2 x 10^5 irradiated PAC from normal or tolerant mice were used to reconstitute NA spleen cells. Cultures were challenged with TNP-Ba (solid bars) or FLU-Ba (hatched bars).

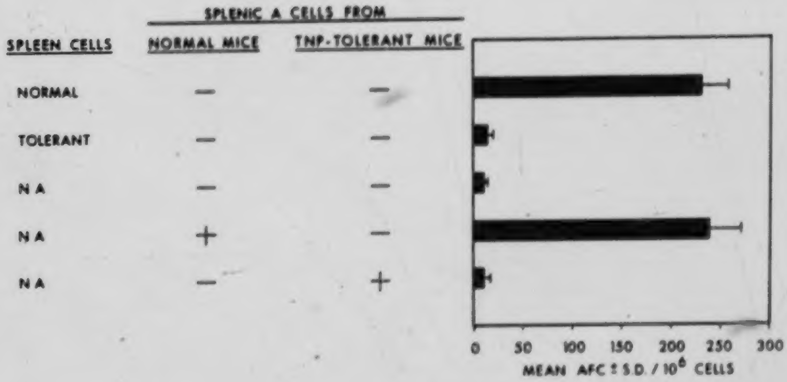


Figure 6. Splenic A cells from tolerant mice fail to reconstitute NA spleen cells. Mice were rendered tolerant by injection of 300 μg TNP$_6$CMC i.v. before use. 10^5 irradiated SAC from normal or tolerant mice were used to reconstitute NA spleen cells. Similar results were observed with from 25 x 10^3 to 3 x 10^5 SAC. Cultures were challenged with TNP-Ba.

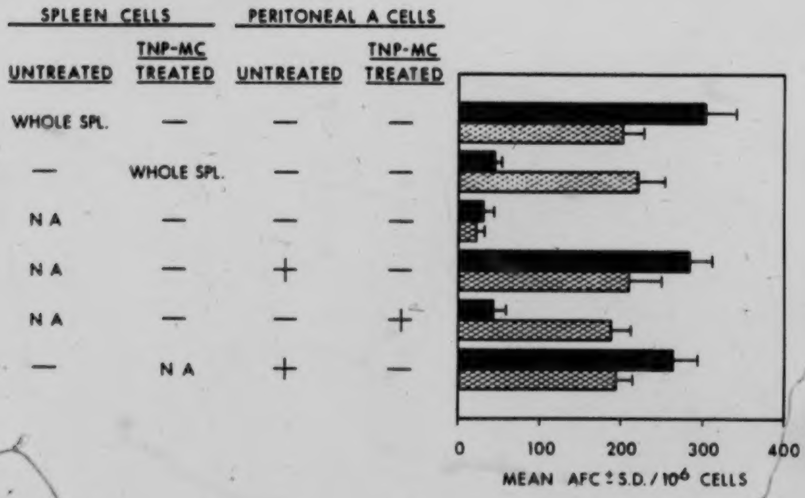


Figure 7. Exposure of PAC to TNP-MC *in vitro* abolishes their capacity to reconstitute immunocompetence in NA spleen cells. Cells were treated with 100 μg/ml TNP$_4$MC at 4° C for 90 min and washed 3 times prior to culture. 2 x 10^5 irradiated untreated or TNP$_4$MC treated PAC were used to reconstitute untreated or TNP$_4$MC treated NA spleen cells. Note: A significant degree of tolerance is not induced in TNP$_4$MC treated NA spleen cells (line 6). Cultures were challenged with TNP-Ba (solid bars) or FLU-Ba (hatched bars).

spleen cells were incubated with TNP-MC at 4° C for 90 min, washed, reconstituted with normal PAC and challenged with TNP-Ba. Such treatment was found to have no effect on B cell responsiveness to TNP (Figure 7). In contrast, similar treatment of unfractionated spleen cells abolished B cell responsiveness to TNP. The above observations suggest that in this system, B cell tolerance is mediated by A cells.

TNP-MC treatment of PAC did not merely shift the dose-response curve of PAC in this system, since TNP-MC treated PAC were unable to restore immunocompetence to TNP-Ba over a wide range of PAC concentrations (Figure 8).

To rigorously test the hapten specificity of this phenomenon, normal and TNP-MC treated spleen cells were simultaneously challenged with TNP-Ba and FLU-Ba in the same culture. The results indicate that strong bystander stimulation by FLU-Ba did not reverse or interfere with the induction of tolerance in TNP specific B cells (Table 1).

To provide direct evidence that A cells, and not other cell types present in PAC or SAC, are responsible for the induction of tolerance in NA spleen cells by TNP-MC, MØ were purified from mouse spleen and their tolerance inducing capability was assessed. As expected, pretreatment with TNP-MC completely abolished their capacity to reconstitute NA spleen cells (Table 2). This suggests that MØ represent one type of A cell which can induce TNP-MC-mediated, hapten specific tolerance in NA cells.

Hapten Specific A Cell Defect is Independent of T Cells

Earlier work has shown that tolerance induced by the hapten carriers CMC or MC is independent of T cells. Homozygous nude mice or adult thymectomized, irradiated and fetal liver reconstituted mice were as susceptible to tolerance induction as normal mice (Diner *et al*, 1979; von Borstel *et al*, 1983). In addition, mixed cultures of normal and tolerant spleen cells yielded no evidence for the presence of suppressor T cells in tolerant mice (Diner *et al*, 1979). The following experiments confirm these earlier studies by showing that the inability of PAC from tolerant mice to reconstitute immunocompetence is independent of T cells. Rigorous T cell depletion was accomplished by ATS treatment *in vivo* followed by treatment *in vitro* with a

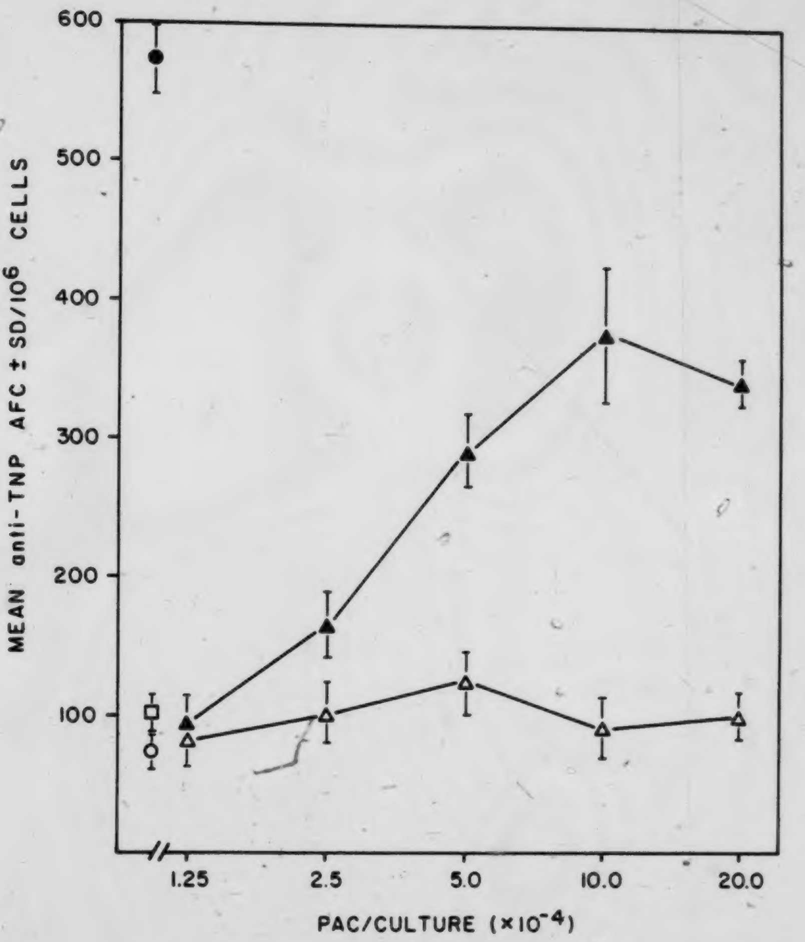


Figure 8. AFC response by A cell deficient spleen cells as a function of the number of normal or TNP-MC treated A cells added in culture. Normal spleen cells treated with RPMI containing 10% FCS alone (closed circle) or TNP₄MC (100 µg/ml for 90 min at 4° C, open circle), and NA spleen cells alone (open square) or reconstituted with normal (closed triangles) or TNP₄MC treated (open triangles) PAC, were cultured with TNP-Ba.

TABLE 1

Failure to Reverse TNP-MC Induced Tolerance by Strong Bystander Stimulation

Spleen Cells	Antigen in Culture		Mean AFC ± SD/10^6 cells	
	TNP-Ba	FLU-Ba	anti-TNP	anti-FLU
Normal	+	–	476 ± 52	<10
Normal	–	+	<10	224 ± 16
Normal	+	+	405 ± 41	218 ± 27
Tolerant	+	–	27 ± 8	<10
Tolerant	–	+	<10	195 ± 16
Tolerant	+	+	34 ± 12	231 ± 19

CBA/CaJ spleen cells were treated with RPMI containing 10% FCS alone (normal) or containing 100 μg/ml TNP_4MC (tolerant) for 90 min at 4° C. Cells were then washed 3 times, and cultured with TNP-Ba and/or FLU-Ba as indicated.

TABLE 2

Exposure of Splenic MØ to TNP-MC *In Vitro* Abolishes Their Capacity to Reconstitute Immunocompetence in NA Spleen Cells

Spleen Cells[a]	Splenic MØ[b]		Mean anti-TNP AFC ± SD /10^6 cells
	No./Culture ($\times 10^{-4}$)	TNP-MC treated	
Normal	0		268 ± 34
NA	0		12 ± 2
NA	2.5	–	180 ± 22
NA	5.0	–	232 ± 27
NA	2.5	+	35 ± 9
NA	5.0	+	46 ± 18

[a] CBA/CaJ normal or NA spleen cells (10^6/culture) were cultured with TNP-Ba.

[b] MØ were purified from CBA/CaJ spleen cells and used to reconstitute NA spleen cells at the indicated concentrations. Where indicated, MØ were treated with 100 μg/ml TNP-MC for 90 min at 4° C and washed 3 times prior to culture.

mixture of anti-Thy-1.2 and anti-Ly-2.1 antibody and C'. Such T cell depletion failed to reverse tolerance induced either *in vivo* or *in vitro* (Table 3). Further, treatment of PAC, from mice rendered tolerant *in vivo* by TNP-MC, with anti-Thy-1.2 antibody and C' failed to restore their ability to cooperate with NA spleen cells in the response to TNP-Ba (Table 4). Treatment of NA spleen cells with anti-Thy-1.2 antibody and C' prior to reconstitution with anti-Thy-1.2 antibody and C' treated PAC from tolerant mice yielded similar results (Table 4). Finally, when mixtures of normal and tolerant PAC were used to reconstitute immunocompetence to TNP-Ba in NA spleen cells, the response was directly proportional to the number of normal PAC in the mixture (Figure 9). These and previously published results (Diner *et al*, 1979; von Borstel *et al*, 1983) strongly suggest that suppressor T cells are not present in the A cell population nor induced in the normal NA population.

TNP-MC Treated A Cells Induce Tolerance in B Cells

The inability of A cell deficient spleen cells to be rendered tolerant by TNP-MC *in vitro* (Figure 7) suggests that tolerance in this system is mediated by A cells. To more directly assess this possibility, NA spleen cells were precultured at 37° C with normal or TNP-MC treated PAC prior to reconstitution with normal PAC and challenge with TNP-Ba. The results show (Table 5) that preincubation with TNP-MC treated PAC for only 9 hr was sufficient to significantly affect subsequent responsiveness to TNP-Ba under normally stimulatory conditions. NA spleen cells preincubated with TNP-MC treated PAC for 18 hr were completely unresponsive upon challenge with TNP-Ba and PAC (Table 5). These data suggest that B cells very rapidly become irreversibly and hapten specifically tolerized by interaction with tolerogen treated A cells. The possible mechanisms by which this may occur is the subject of *Chapter VI*.

D. Discussion

We have demonstrated that radioresistant Thy-1^-, Ly-2^- A cells from normal or ATS treated mice rendered hapten specifically tolerant by hapten derivatized CMC or MC fail to reconstitute an *in vitro* response by normal NA cells to the tolerizing hapten conjugated to Ba.

TABLE 3

In Vivo and *In Vitro* Induced Tolerance is Not Mediated by T Cells

Treatment of Spleen Cells		Mean anti-TNP AFC ± SD/10^6 cells
In vivo[a]	*In vitro*[b]	
–	–	433 ± 55
TNP-CMC	–	53 ± 22
TNP-CMC, ATS	–	61 ± 16
TNP-CMC, ATS	C'	47 ± 18
TNP-CMC, ATS	αThy-1.2, αLy-2.1, C'	58 ± 12
ATS	–	403 ± 88
ATS	TNP-MC	68 ± 8
ATS	C'	361 ± 30
ATS	αThy-1.2, αLy-2.1, C'	216 ± 37
ATS	αThy-1.2, αLy-2.1, C', TNP-MC	48 ± 11

[a] Mice were rendered tolerant by i.v. injection of 300 μg TNP_6CMC 48 hr prior to assay. ATS was injected i.v. alone or together with TNP_6CMC 48 hr prior to assay.

[b] Spleen cells from ATS pretreated normal or tolerant mice were treated with αThy-1.2 and αLy-2.1 for 90 min at 4° C followed by rabbit C' for 45 min at 37° C. Cells were washed 3 times before further treatment or culture. AFC control response to chicken red blood cells confirmed the effectiveness of ATS treatment αThy-1.2, αLy-2.1 and C' treatments: spleen cells treated with C' alone, 100 ± 18 AFC/10^6 cells: spleen cells treated with αThy-1.2, αLy-2.1 and C', 5 ± 2 AFC/10^6 cells. Normal or αThy-1.2, αLy-2.1 and C' treated spleen cells from ATS pretreated mice were incubated with 100 μg/ml of TNP_4MC for 90 min at 4° C and washed 3 times before culture. Cultures were challenged with TNP-Ba.

TABLE 4

Failure of A Cells From Tolerant Mice to Reconstitute A Cell Deficient Spleen Cells is Independent of Thy-1$^+$ Cells

Spleen Cells	Reconstituted with 2 x 10^5 A Cells from			Mean anti-TNP
	normal mice	TNP-MC tolerant mice[a]	TNP-CMC tolerant mice[a]	AFC ± SD/10^6 cells
Normal	–	–	–	412 ± 159
NA	–	–	–	68 ± 14
NA	αThy-1.2, C'	–	–	332 ± 84
NA	C'	–	–	292 ± 40
NA (αThy-1.2, C')	+	–	–	252 ± 64
NA (αThy-1.2, C')	αThy-1.2, C'	–	–	308 ± 48
NA (C')	C'	–	–	412 ± 92
NA	–	+	–	92 ± 24
NA	–	αThy-1.2, C'	–	88 ± 48
NA	–	C'	–	80 ± 24
NA (αThy-1.2, C')	–	+	–	68 ± 12
NA (αThy-1.2, C')	–	αThy-1.2, C'	–	84 ± 16
NA (αThy-1.2, C')	–	–	+	88 ± 24
NA (αThy-1.2, C')	–	–	αThy-1.2, C'	72 ± 28

[a] A cells from tolerant mice were obtained 48 hr after injection of 300 μg of the tolerogen TNP_4MC or TNP_6CMC. Cultures were challenged with TNP-Ba.

TABLE 5

TNP-MC Pulsed PAC Induce TNP Specific Tolerance in A Cell Deficient Spleen Cells

Spleen Cells	Precultured[a] with 2 x 10^6		No. PAC/Culture (x 10^{-4})	Mean anti-TNP AFC ± SD /10^6 cells
	PAC	PAC (TNP-MC)		
Normal	–	–	–	520 ± 58
(A) NA	+	–	0	35 ± 15
NA	+	–	5	351 ± 52
NA	+	–	10	318 ± 41
NA	–	+	0	23 ± 12
NA	–	+	5	98 ± 20
NA	–	+	10	78 ± 16
(B) NA	+	–	0	22 ± 8
NA	+	–	5	226 ± 29
NA	+	–	10	200 ± 12
NA	–	+	0	<10
NA	–	+	5	<10
NA	–	+	10	<10

[a] For the preculture phase, 2 x 10^6 CBA/CaJ PAC were cultured in 12 well Linbro trays in 2 ml RPMI containing 10% FCS with or without 100 μg/ml TNP$_4$MC at 37° C. After 2 hr, nonadherent peritoneal cells were resuspended by rocking and discarded. The remaining monolayers were washed 3 times with warm medium, followed by the addition of 2 x 10^7 CBA/CaJ NA spleen cells in 2 ml RPMI with 10% FCS. After 9 (A) or 18 (B) hr, NA spleen cells were resuspended by rocking, removed, and washed 3 times. The precultured NA cells were then cultured with TNP-Ba plus the indicated numbers of PAC as usual.

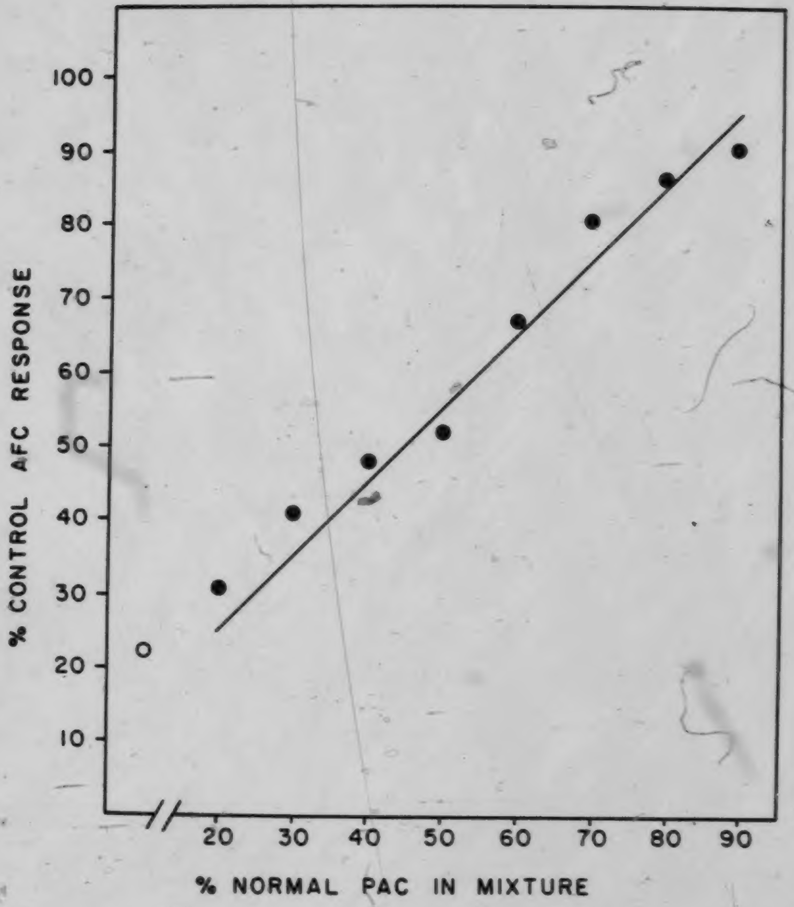


Figure 9. Reconstitution of NA spleen cells with various mixtures of normal and TNP-MC treated PAC. CBA/CaJ NA spleen cells alone (open circle) or reconstituted with the indicated mixtures of normal plus TNP$_4$MC treated PAC (10^5 total, closed circles) were cultured with TNP-Ba. Results are expressed as the percent of the TNP specific AFC response in NA spleen cells reconstituted with 10^5 normal PAC (175 ± 25 AFC/10^6 cells).

A cells from tolerant mice however, were found capable of cooperating in an *in vitro* response to a second unrelated hapten. These findings confirm and expand previous observations that radioresistant Thy-1^- A cells from mice rendered tolerant to POL failed to restore immunocompetence to that antigen, but could augment the *in vitro* response to SRBC (Diener *et al*, 1976, 1977).

The most intriguing aspect of our observation is the hapten specificity of the A cell deficiency in tolerant mice. To account for this unusual phenomenon, we first considered the involvement of suppressor T cells. MØ which have absorbed antigen specific T_s factor ("armed" MØ) have been shown to hapten specifically suppress the transfer of contact sensitivity in mice (Zembala and Asherson, 1974). Subsequently, however, it was demonstrated that the suppression only appeared hapten specific; mice simultaneously challenged with the relevant and an irrelevant hapten were nonspecifically suppressed (Asherson and Zembala, 1974). Thus, the "armed" MØ were hapten specifically triggered to release a nonspecific mediator (Ptak *et al*, 1978), analagous to the antigen specific triggering of histamine release by cytophilic IgE on mast cells. Such a phenomenon cannot explain the hapten specificity of A cell mediated tolerance in our system, since spleen cells simultaneously challenged with the tolerizing and an irrelevant hapten responded normally to the irrelevant hapten (Table 1).

Additional evidence against the involvement of suppressor T cells in the establishment and the control of tolerance induced by hapten conjugates of CMC or MC is provided by the following observations: 1) We have demonstrated in previously published work that tolerance induced by hapten conjugates of CMC or MC is equally effective in homozygous nude mice, in thymectomized, irradiated, fetal liver-reconstituted mice and in normal mice (Diner *et al*, 1979; von Borstel *et al*, 1983); 2) Rigorous depletion of T cells by treatment *in vivo* with ATS followed by treatment *in vitro* of spleen cells with anti-Thy-1 and anti-Ly-2 antibody and C' failed to affect tolerance induction *in vitro* by hapten derivatized MC or to restore responsiveness in spleen cells from TNP-CMC treated mice; 3) When mixtures of normal and tolerizing A cells were used to reconstitute immunocompetence in NA spleen cells to TNP-Ba, the response was directly proportional to the number of normal A cells in the mixture (Figure

9). Finally, the use of different carriers for tolerance induction and for immunogenic challenge further precludes a carrier specific suppressive effect by regulatory T cells.

Adherent accessory cells are required for the induction of both T cell dependent and T cell independent B cell responses (Mosier, 1967; Lee *et al*, 1976). However, our current state of knowledge regarding A cell-B cell interaction does not provide a ready explanation for hapten specific unresponsiveness at the level of the A cell. T cell derived soluble immune response suppressor factor (SIRS) reported by Pierce and colleagues cannot be relevant to our experimental model because SIRS is T cell dependent and antigen nonspecific (Tadakuma and Pierce, 1976). Furthermore, it is unlikely that tolerizing A cells release long range suppressive molecules, since TNP tolerant spleen cells simultaneously challenged with TNP-Ba and FLU-Ba in the same culture, exhibited a normal FLU specific response (Table 1). This is consistent with the notion that putative A cell derived short range factors act on B cells in close association with A cells. Thus, tolerance would only be induced to those haptens presented in conjunction with the tolerogenic carriers. This possibility is directly assessed in *Chapter VI*.

The data at hand are so far suggestive of a causal relationship between hapten specifically tolerizing A cells and the functional (or physical) deletion of B cells. Within a short time of interaction between normal B cells and tolerizing A cells, the former were rendered unresponsive, even when the tolerizing A cells were subsequently replaced by normal A cells.

In order to assess the mechanism(s) by which tolerogen treated A cells render B cells tolerant to a TI antigen challenge, the functions of A cells in the activation of nontolerant B cells by TI antigens must be considered. Surprisingly, such functions have not been well investigated. Therefore, the studies in the following chapter were undertaken to clarify this point, in order that the mechanism of A cell induced B cell tolerance might be more readily investigated.

V. Accessory Cell Function in Thymus Independent B Cell Activation

A. Introduction

The induction of antigen specific T cell mediated immune responses and thymus dependent B cell responses is critically dependent on A cells such as MØ and dendritic cells DC (reviewed in Steinman, 1981; Unanue, 1984). Accessory cell functions include antigen processing (reviewed in Unanue, 1984), MHC restricted presentation of antigens in the context of class II MHC molecules (Ia antigens) (reviewed in Schwartz *et al*, 1978; Rock and Benacerraf, 1983), and IL-1 secretion (reviewed in Durum *et al*, 1985). B cell activation by TI antigens also requires A cells (Chused *et al*, 1976; Lee *et al*, 1976; Pierce and Kapp, 1976a; Boswell *et al*, 1980c; Letvin *et al*, 1981; Morrissey *et al*, 1981; Corbel and Melchers, 1983) but their precise functions have not been defined. Recent evidence that B cell responses to some TI antigens and mitogens are dependent on T cell derived nonspecific factors (Mond *et al*, 1980; Letvin *et al*, 1981; Jaworski *et al*, 1982; Zubler and Glasebrook, 1982; Endres *et al*, 1983; Nossal and Pike, 1984; Pike and Nossal, 1984; Pike *et al*, 1984) has lead to speculations that A cells mediate identical functions in T cell activation and TI B cell activation (Boswell *et al*, 1980c; Letvin *et al*, 1981; Morrissey *et al*, 1981). A cells or their soluble products could thus be acting indirectly in TI AFC responses *via* the induction of T cell factors. Alternatively, direct A cell-B cell interactions may be required.

Evidence for the latter possibility has been obtained by Morrissey *et al* (1981), who showed that the *in vitro*, TNP specific, AFC response by A cell deficient spleen cells was reconstituted by Ia$^+$, SAC pulsed with TNP-ficoll. Letvin *et al* (1981) reported similar results, but in view of the requirement for ficoll specific T_h in their system, A cell-T cell interactions could not be ruled out. Singer and Hodes (1982) and Hodes *et al* (1983a,b) further investigated A cell-B cell interactions in AFC responses to TNP-ficoll under TD and TI conditions. In both cases, direct MHC restricted A cell-B cell interactions were implicated. Evidence of such interactions has also been obtained in TD systems, in which A cell-T cell restriction was not evident (Nisbet-Brown *et al*, 1981), or in which the helper signal was provided by LPS

(Gorczynski *et al*, 1980).

Despite the implications of these studies, and suggestions of Boswell *et al*, 1980c, Morrissey *et al* (1981), and Letvin *et al* (1981) that A cells may process and present TI antigens in the context of Ia molecules to T and/or B cells, this has not been directly investigated. In view of this, we used several A cell populations, including homogeneous MØ-like and DC-like tumor lines, with different cell surface and functional characteristics, to directly investigate A cell functions in antibody forming cell responses to TI-1 and TI-2 antigens. These antigens are distinguished according to their relative immunogenicity in CBA/N mice (Mond *et al*, 1978; Mosier and Subbarao, 1982). We show that in contrast to T cell activation, A cell function in TI B cell activation does not involve MHC restricted presentation of processed antigens, and can be entirely replaced by pure recombinant IL-1.

B. Materials and Methods

Mice. C3H/HeJ (H-2^k) mice, 4 to 6 wk old, were obtained from the Ellerslie Small Animal Farm, University of Alberta, Edmonton, Alberta.

Antigens. TNP conjugates of Ba, LPS, ficoll, and dextran were prepared as described in *Chapter III*. TNP-Ba organisms were used at 14 x 10^8/culture. TNP-LPS, TNP-ficoll, and TNP-dextran were used at 100 ng/ml.

Antibodies. I-A^k specific (10-2.16) (Oi *et al*, 1978) and I-A^d specific (MKD6) (Kappler *et al*, 1981) B cell hybridomas were obtained from the American Type Culture Collection and adapted to grow in serum-free DSI medium (Quadralogic Co., Vancouver, B.C.). The optimal (saturating) binding activity of cell-free supernatants was determined by an enzyme linked immunosorbent assay (Warren and Vogel, 1985).

Accessory Cells. PAC and SAC were obtained as described in *Chapter III*.

Culture derived bone marrow-macrophages (BM-MØ) were grown in the presence of L cell conditioned medium as a source of MØ growth factor according to Lee and Wong (1982). Adherent cells harvested on day 6 (100% Fc receptor positive phagocytic MØ) were fractionated according to size by the velocity sedimentation technique of Miller and Phillips as modified for

MØ (Figure 10), (Lee and Berry, 1977). The fractionated BM-MØ were pooled into 5 fractions (A to E) and activated with supernatant from Con A stimulated rat spleen cells (Glasebrook and Fitch, 1980, 10% v/v) plus 3 μg/ml LPS overnight as described (Lee and Wong, 1982). The following day, the medium was removed and the cells were washed 3 times with warm DSI medium. Antigen and NA cells were then added in DSI medium. This protocol is summarized in Figure 10.

The DBA/2 A cell lines P388AD.2 and P388NA.10 (courtesy of Dr. D.A. Cohen, University of Kentucky) and $P388D_1$ (American Type Culture Collection) were grown in RPMI medium supplemented with 10% fetal calf serum ("Rehatuin", Reheis Chemical Co., Phoenix, AZ). Accessory cell tumor lines were treated with 25 μg/ml mitomycin C (Sigma, St. Louis, MO) for 45 min at 37° C and washed 3 times prior to culture.

Helper T Cell Line. A keyhole limpet hemocyanin (KLH) specific T cell line ($CKA\text{-}T_h$) was derived by the procedure of Kimoto and Fathman (1980). Briefly, popliteal lymph nodes were removed from CBA/CaJ mice which had been immunized in the hind footpads with 0.1 ml of an emulsion containing equal proportions of KLH (20 μg) (Calbiochem-Behring, La Jolla, CA) in saline and Complete Freund's Adjuvant (0.5 mg/ml heat killed *Mycobacterium tuberculosis* H37Ra in mineral oil) (Difco Laboratories, Detroit, MI). Single cell suspensions were prepared and passed over nylon wool (see below) to obtain a T lymphocyte enriched, A cell and B cell depleted population. T cells (2×10^6/ml) were then cultured with irradiated (1500 rad) syngeneic spleen cells (1×10^6/ml) and 50 μg/ml KLH in 1 ml RPMI containing 10% FCS and 5×10^{-5} M 2-ME in Linbro 24-well tissue culture trays at 37° C in a 10% CO_2/air humidified atmosphere. After 3-4 days, cells were harvested, washed with RPMI and 10% FCS, layered over ficoll-hypaque (density 1.09 g/cm^3), and centrifuged at 800 *g* for 25 min at 20° C. Blast cells were recovered at the ficoll-medium interface, washed, and recultured (10^5/ml) with irradiated spleen cells (2.5×10^6/ml), but without KLH, in 2 mls RPMI with 10% FCS and 5×10^{-5} M 2-ME in Linbro 24-well tissue culture trays at 37° C. After 10-14 days, cells were harvested and the live cells (recovered by centrifugation over ficoll) recultured under the same conditions as above, except that 50 μg/ml KLH was included.

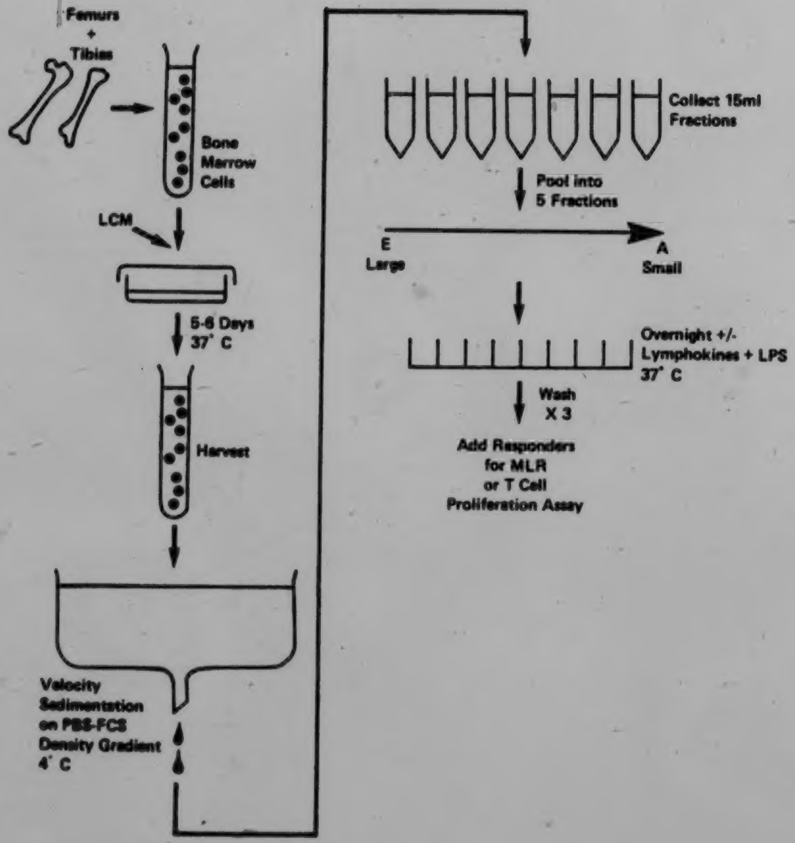

Femurs
+
Tibias
Bone
Marrow
Cells
LCM
5-6 Days
37° C
Harvest
Velocity
Sedimentation
on PBS-FCS
Density Gradient
4° C
Collect 15ml
Fractions
Pool into
5 Fractions
E
Large
A
Small
Overnight +/-
Lymphokines + LPS
37° C
Wash
X 3
Add Responders
for MLR
or T Cell
Proliferation Assay

Figure 10. Protocol for growth and size-fractionation of BM-MØ.

This cycle of antigenic stimulation (for 3-4 days) and resting periods without antigen (10-14 days) was continued indefinitely (Figure 11).

Nylon Wool Purification of T Cells. Before use, the nylon wool (Fenwall Laboratories Inc., Calgary, Alta.) was autoclaved with 0.1 N HCl and then washed by autoclaving 6 times with double distilled water, after which it was thoroughly air dried for 1 wk. Nylon wool columns were prepared by packing 2.5 g of dry nylon wool into the barrel of a 20 ml disposal syringe and autoclaving for sterility.

Lymph node cells were nylon wool filtered by a modification of the technique described by Julius *et al* (1973). The packed sterile column was filled with warm (37° C) RPMI containing 5% FCS and allowed to drain while long, sterile forceps were used to squeeze out air bubbles. Once the medium reached the top of the nylon wool, lymph node cells (no more than 2.5 x 10^8 in 4 ml warm medium) were slowly added to the top of the column. Once the cells had entered the column, 2 ml warm medium were added and allowed to enter the column. After 45-60 min incubation at 37° C, the column was washed with 40-50 ml warm RPMI containing 5% FCS and the T cell enriched effluent collected.

Proliferation Assay. Six to eight hr before harvesting, 0.625 μCi of [methyl-^{3}H] thymidine (specific activity 20 Ci/mmol, Amersham Corp., Oakville, Ont.) in 0.025 ml RPMI was added to each well. Cells were harvested onto Titertek filter paper with a Titertek semiautomated cell harvester (Flow Laboratories, Inglewood, CA). ^{3}H-thymidine uptake was determined by liquid scintillation spectrometry, and the results expressed as mean cpm ± SD of triplicate cultures.

Interleukin-1. Pure recombinant murine IL-1 was kindly provided by Dr. Peter Lomedico of Hoffmann-La Roche Inc., Department of Molecular Genetics, Roche Research Center, Nutley, NJ (Lomedico *et al*, 1984). Activity was assessed by a thymocyte proliferation assay (Lee *et al*, 1981) (Figure 12). Briefly, thymocytes (5 x 10^4/well) from 4-6 wk old C3H/HeJ mice were cultured with or without 6 μg/ml Con A (Calbiochem, La Jolla, CA) and with various concentrations of IL-1 in RPMI 1640 supplemented with 10% FCS and 5 x 10^{-5} M 2-ME in 96-well round bottomed microtiter trays. ^{3}H-thymidine uptake was determined on

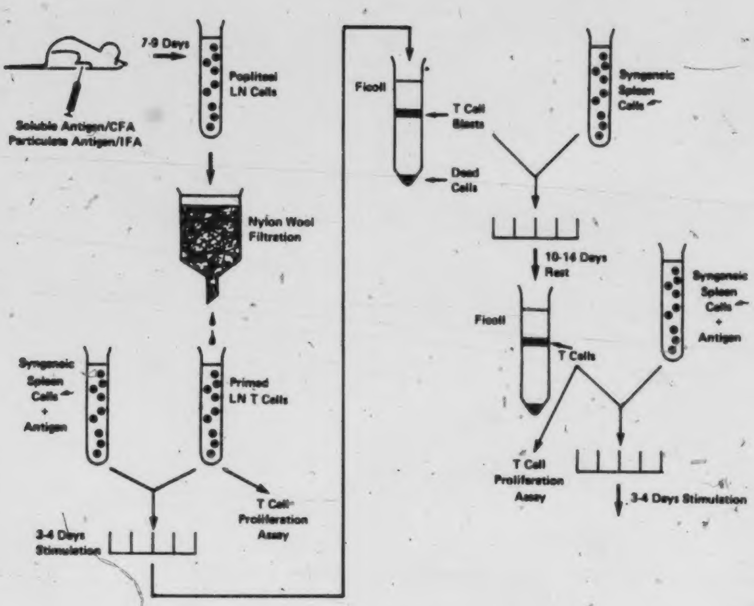

7-9 Days
Popliteal LN Cells
Soluble Antigen/CFA
Particulate Antigen/IFA
Nylon Wool Filtration
Syngeneic Spleen Cells
+
Antigen
Primed LN T Cells
T Cell Proliferation Assay
3-4 Days Stimulation
Ficoll
T Cell Blasts
Dead Cells
Syngeneic Spleen Cells
10-14 Days Rest
Ficoll
T Cells
Syngeneic Spleen Cells
+
Antigen
T Cell Proliferation Assay
3-4 Days Stimulation

Figure 11. Protocol for the generation of antigen specific T cell lines.

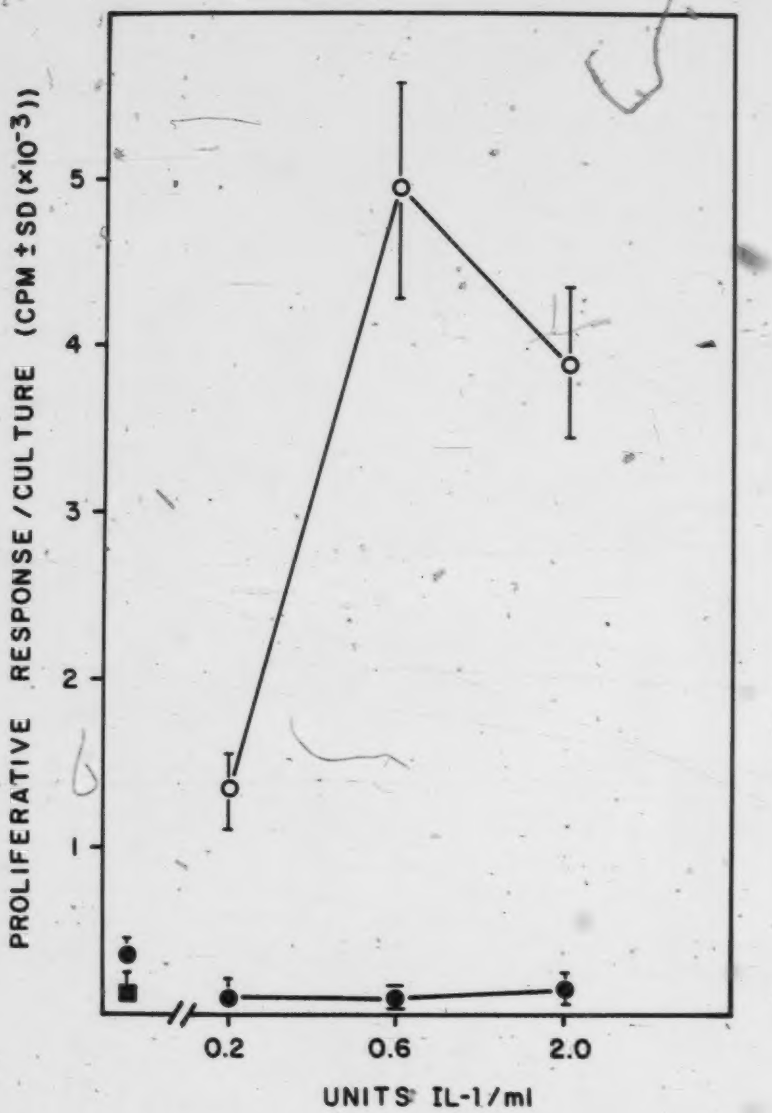


Figure 12. Thymocyte proliferative response to recombinant IL-1. Thymocytes from 4-6 wk old C3H/HeJ mice were cultured with (open circles) or without (closed circles) 6 μg/ml Con A (Calbiochem, La Jolla, CA) and with various concentrations (U/ml) of IL-1. Proliferative responses of thymocytes cultured alone (closed circle) or with Con A (closed square) in the absence of IL-1 were also assessed.

day 3.

Flow Cytometry. Cells in V-bottomed microtiter wells (5 x 10^5 to 10^6/well) were pretreated for 1 hr at 4° C with 0.2% Bacto gelatin (Difco Laboratories, Detroit, MI) in Hanks balanced salt solution (HBSS, 0.1 ml/well) to block nonspecific binding. The medium was then replaced with 0.1 ml of 10-2.16 or MKD6 serum-free culture supernatant diluted 100 times in HBSS containing 0.1% gelatin and 0.02% azide. This was followed by 0.1 ml of affinity purified fluorescein conjugated sheep anti-mouse IgG $F(ab')_2$ (Cappel, Cochraneville, PA) diluted 50 times in the same medium. Both incubations were for 45 min at 4° C with three washes after each incubation. Following the last wash, the cells were fixed in 2% formaldehyde-PBS and analyzed immediately or on the next day with an EPICS V fluorescence activated cell sorter (FACS) (Coulter Electronics, Hialeah, FL) using an argon laser exciting at 488 nm, a 492-502 nm blocking filter, and a 525 nm bandpass filter. Fluorescence (log amplification) data were collected with the gates for forward angle light scatter adjusted to exclude dead cells and debris, and analyzed on the Coulter MDADS computer.

Tissue Culture. Tissue culture was carried out as described in *Chapter III*, except that DSI serum-free medium (Quadralogic Co., Vancouver, B.C.) was used for these studies. Neither the kinetics, nor the magnitude of AFC responses differed significantly from those obtained in RPMI supplemented with 10% FCS and 5 x 10^{-5} M 2-ME. Serum-free medium was used to avoid possible obscurement of IL-1 dependence by FCS components (Hoffmann *et al*, 1984).

C. Results

A Cell Dependence of AFC Responses to TI-1 and TI-2 Antigens

We have previously demonstrated a strict A cell dependence for AFC responses to the TI-1 antigen TNP-Ba (Diner *et al*, 1986). However, there have been numerous reports demonstrating both a requirement (Lee *et al*, 1976; Mond, 1982; Corbel and Melchers, 1983; Fernandez and Severinson, 1983) or lack thereof (Yoshinaga *et al*, 1972; Lemke *et al*, 1975;

Wong and Herscowitz, 1979; Boswell *et al*, 1980a,b; Morisaki *et al*, 1983) for A cells in AFC responses to some TI antigens and mitogens. In an attempt to resolve this conflict, spleen cells were rigorously depleted of A cells and assessed for *in vitro* AFC responses to both TI-1 and TI-2 antigens. A cell depleted NA spleen cells were unable to generate primary *in vitro* AFC to the TI-1 antigens TNP-Ba and TNP-LPS, as well as to the TI-2 antigens TNP-ficoll and TNP-dextran (Figure 13). Syngeneic PAC restored the TNP specific AFC responses to these four TI antigens in a dose dependent manner (Figure 13). SAC also restored immunocompetence to TNP-Ba in NA spleen cells, and were more efficient than PAC on a per cell basis (Figure 14).

Since A cells display functional heterogeneity for T cell activation (Lee, 1980; Lee and Wong, 1980, 1982; Guidos *et al*, 1984), various sources of A cells were compared for the ability to restore A cell dependent AFC responses to TNP-Ba. Absolutely pure MØ were grown from low numbers of bone marrow precursors in the presence of MØ growth factor (Lee and Wong, 1980), harvested after 6 days and fractionated according to size (Lee and Berry, 1977) and used to reconstitute the AFC response to TNP-Ba in NA spleen cells (Figure 15). Activation with lymphokines and LPS to induce Ia expression (Lee and Wong, 1982) was not necessary for A cell function in this system. Moreover, all size fractions of BM-MØ were functional to some extent, although high concentrations of the larger, activated BM-MØ were suppressive (Figure 15, left panel). Consistent with this, a preponderance of nonspecific suppressive activity in large BM-MØ populations has been demonstrated in T cell activation (Lee and Wong, 1982) and in B cell activation by a TI antigen (Diener *et al*, 1970).

These results demonstrate that *in vitro* AFC responses to both TI-1 and TI-2 antigens exhibit a strict dependence on A cells, provided that rigorous A cell depletion methods have been employed. Moreover, a number of functionally distinct A cell types are competent to restore responsiveness to these antigens in A cell deficient spleen cells.

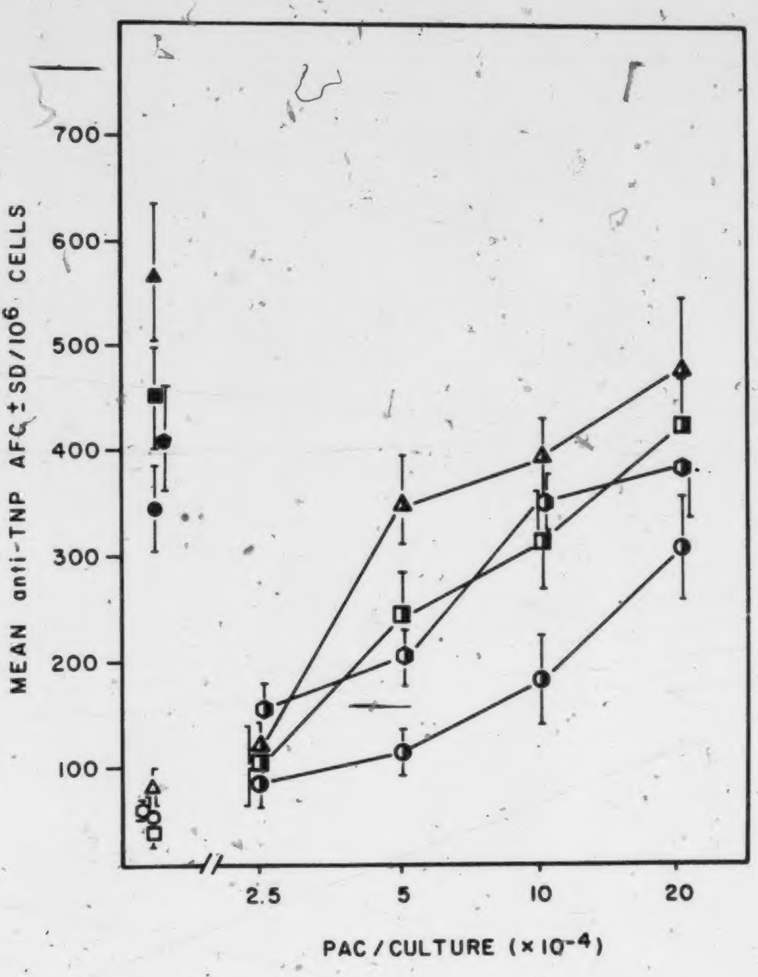


Figure 13. A cell dependence of AFC responses to TI antigens. CBA/CaJ normal spleen cells (closed symbols), NA spleen cells (open symbols), or NA spleen cells plus graded numbers of PAC (half-closed symbols), were cultured with TNP-LPS (triangles), TNP-ficoll (squares), TNP-dextran (circles), or TNP-Ba (hexagons).

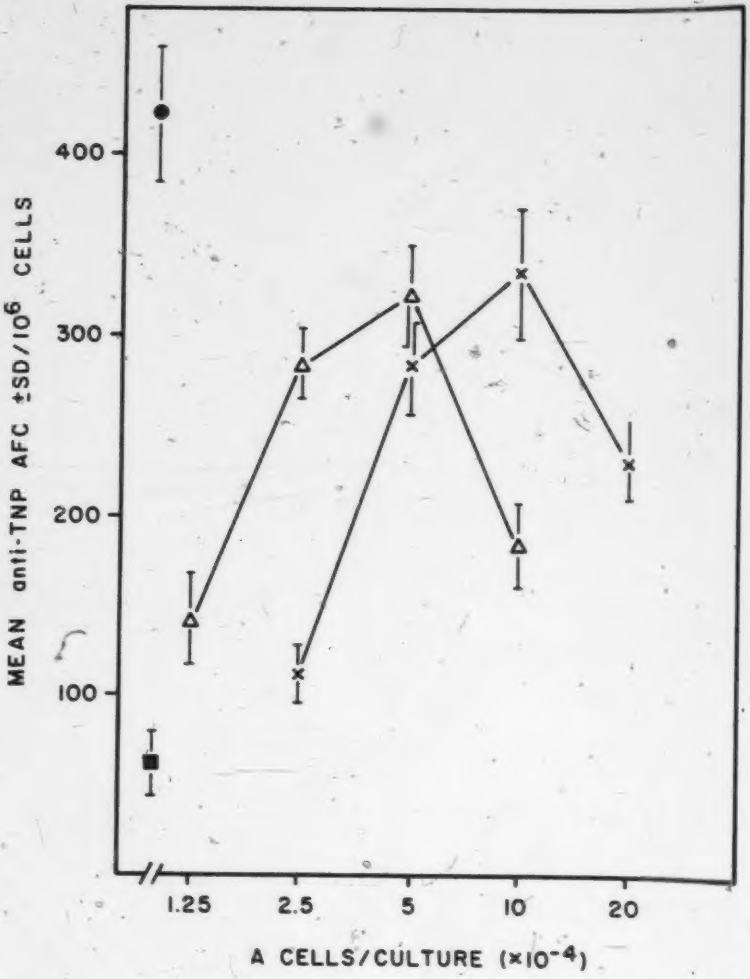


Figure 14. Comparison of PAC and SAC for reconstitution of the AFC response to TNP-Ba. CBA/CaJ normal (closed circle), NA (closed square) spleen cells, and NA spleen cells reconstituted with graded numbers of PAC (x) or SAC (open triangles), were cultured with TNP-Ba organisms.

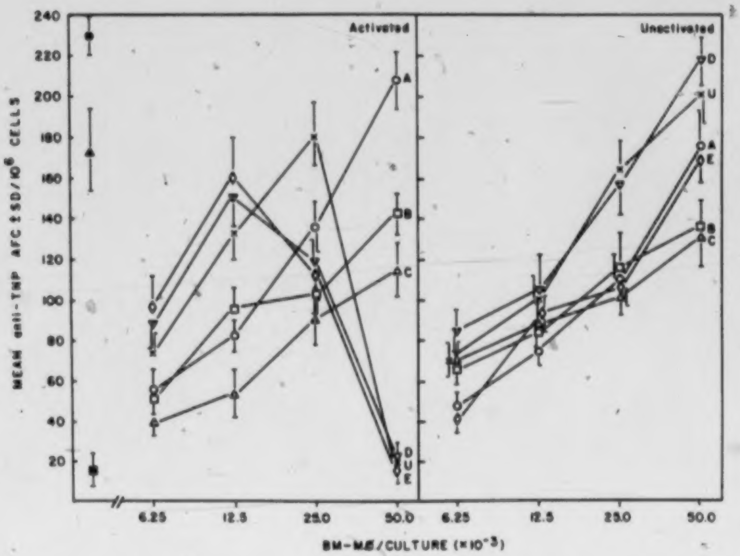


Figure 15. Activated and unactivated BM-MØ restore responsiveness to TNP-Ba in A cell deficient spleen cells. Normal (closed circle) or NA (closed square) CBA/CaJ spleen cells were cultured with TNP-Ba. NA spleen cells were reconstituted with 10^5 CBA/CaJ PAC (closed triangle) or graded numbers of CBA/CaJ activated (left panel) or unactivated (right panel) BM-MØ separated according to size by velocity sedimentation and pooled into five fractions (smallest, A, to largest, E), or left unfractionated (U). Sedimentation velocities in mm/hr were: A, 2.6-6.0; B, 6.0-7.9; C, 7.9-9.9; D, 9.9-11.9; and E, 11.9-14.6.

Comparison of A Cell Functions in T Cell Activation and TI B Cell Activation

T cells are activated by the recognition of antigen (usually "processed") on syngeneic antigen presenting cells (APC) (reviewed in Unanue, 1984; Schwartz, 1985). This reflects the dual specificity of T cells for antigen and MHC (reviewed in Bevan and Fink, 1978; Zinkernagel, 1978). If the A cell requirement in TI B cell activation is solely a consequence of the A cell functions required for T cell activation, then A cells should be functionally indistinguishable in the two types of response. Therefore, we undertook a functional comparison of A cells in T cell activation and TI B cell activation.

The requirement for antigen processing in T cell activation was demonstrated using the lysosomotropic agent chloroquine to interfere with lysosome dependent processing steps (Ziegler and Unanue, 1981, 1982; Lee *et al*, 1982; Guidos *et al*, 1984). We assessed the requirement for antigen processing in AFC responses to TI antigens by pretreatment of SAC with chloroquine under conditions which completely abrogated the ability of SAC to process and present KLH for T cell activation (Table 6). Chloroquine treated SAC were as effective as untreated SAC in reconstituting the TNP specific AFC response by NA spleen cells to TNP-Ba (Figure 16). The choloroquine insensitivity of A cell function in these TI B cell responses contrasts with the requirement for processing of most antigens by A cells for T cell activation (reviewed in Unanue, 1984). However, fixation or ultraviolet (UV) irradiation of SAC, which prevent antigen processing and IL-1 secretion (DeFreitas *et al*, 1983; Scala and Oppenheim, 1983), completely abrogated A cell function in this system (Figure 16).

In further contrast to T cell activation, the TNP specific AFC responses to TI antigens did not depend on MHC restricted interaction between NA spleen cells and A cells. Allogeneic and syngeneic PAC were equally effective in reconstituting the TI AFC responses by NA spleen cells (Figure 17). Furthermore, monoclonal anti-I-A^k antibody had no effect on the ability of CBA/CaJ (H-2^d) PAC to reconstitute DBA/2 (H-2^d) NA spleen cells (Table 7). However, in agreement with previous reports, the same antibody significantly inhibited A cell dependent T cell activation (Figure 18). Consistent with these observations, we found no requirement for the expression of Ia antigens by A cells functioning in TI AFC responses. Thus, P388D$_1$, an

TABLE 6

Effect of Various Treatments on A Cell Antigen Presentation Functions in T Cell Activation

A Cell Treatment	IL-1	Proliferative Response (mean Δcpm ± SD/culture ($x\ 10^{-3}$))
medium	–	33.5 ± 4.8
chloroquine	–	2.7 ± 1.0
glutaraldehyde	–	2.0 ± 0.9
glutaraldehyde	+	23.0 ± 2.6
UV	–	2.8 ± 0.7
UV	+	25.7 ± 1.9

CBA/CaJ SAC (3 x 10^5/culture) were pulsed with 100 μg/ml KLH for 1.5 hr at 37° C, washed 3 times, and cultured with a KLH specific T cell line, CKA-T_h, (10^4/culture). Responses to unpulsed SAC have been subtracted. SAC were treated with medium or chloroquine (0.3 mM) for 1.5 hr at 37° C prior to pulsing with KLH (Guidos *et al*, 1984). Glutaraldehyde (30 s in PBS, followed by 0.2 M lysine to neutralize, Shimonkevitz *et al*, 1983) and UV (837 microwatts/cm² for 5 min, DeFreitas *et al*, 1983) treatments were performed after pulsing with KLH. Where indicated, recombinant IL-1 (0.6 U/ml) was included. Proliferative responses were assessed on day 3.

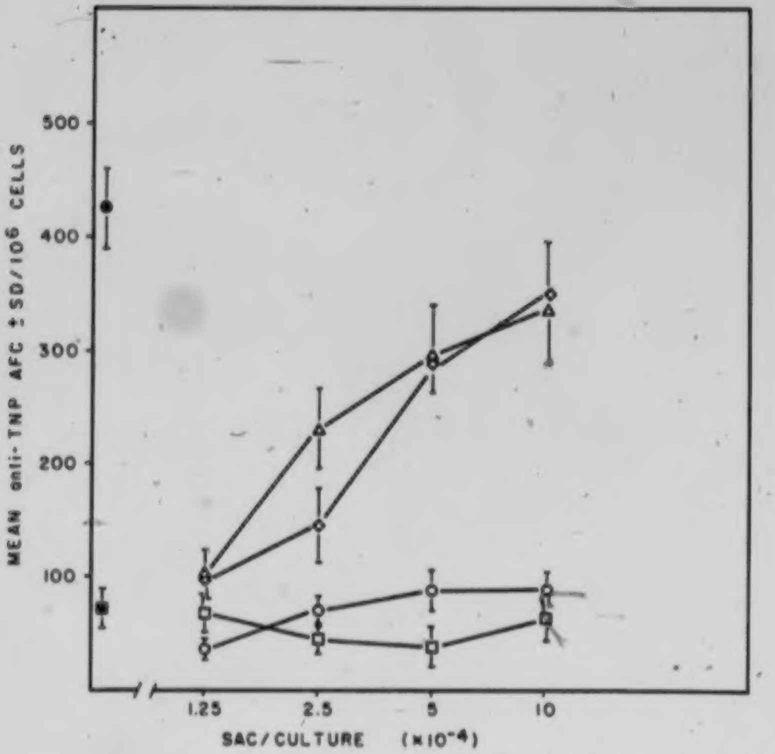


Figure 16. Assessment of A cell antigen presentation function in TI B cell activation. CBA/CaJ normal spleen cells (closed circle), NA spleen cells (closed square), or NA spleen cells plus graded numbers of SAC (open symbols) were cultured with TNP-Ba. SAC were untreated (open diamonds) or treated with chloroquine (open triangles), glutaraldehyde (open squares), or UV (open circles), as described in the legend for Table 6). The effectiveness of these treatments on SAC function in T cell activation is shown in Table 6.

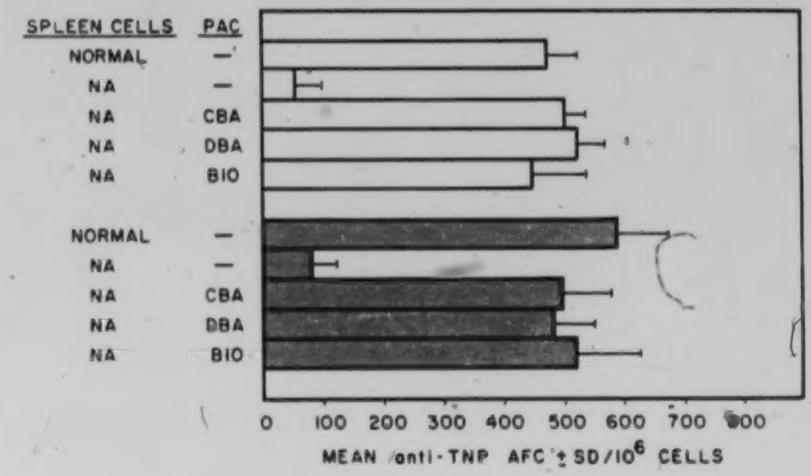


Figure 17. Lack of requirement for MHC restriction. CBA/CaJ NA spleen cells were reconstituted with 10^5 CBA/CaJ (H-2^k), DBA/2 (H-2^d), or C57BL/10 (B10) (H-2^b) PAC and cultured with TNP-Ba (open bars) or TNP-ficoll (hatched bars).

TABLE 7

Failure of Monoclonal Anti-I-A^k Antibody to Block A Cell Function in TI B Cell Activation

DBA/2 Spleen Cells	No. CBA/CaJ PAC/Culture ($x\ 10^{-4}$)	Mean anti-TNP AFC ± SD/10^6 cells	
		No Antibody	αI-A^k
Normal	0	224 ± 29	-
NA	0	44 ± 8	-
NA	1.25	97 ± 23	106 ± 3
NA	2.5	170 ± 8	164 ± 23
NA	5.0	206 ± 24	214 ± 20
NA	10.0	216 ± 35	226 ± 10

DBA/2 NA spleen cells were reconstituted with the indicated numbers of CBA/CaJ PAC in the presence or absence of 10-2.16 (αI-A^k) culture supernatant (1/1000 final dilution). This concentration of αI-A^k significantly blocked antigen presentation by CBA/CaJ spleen cells (see Figure 18). Cultures were challenged with TNP-Ba.

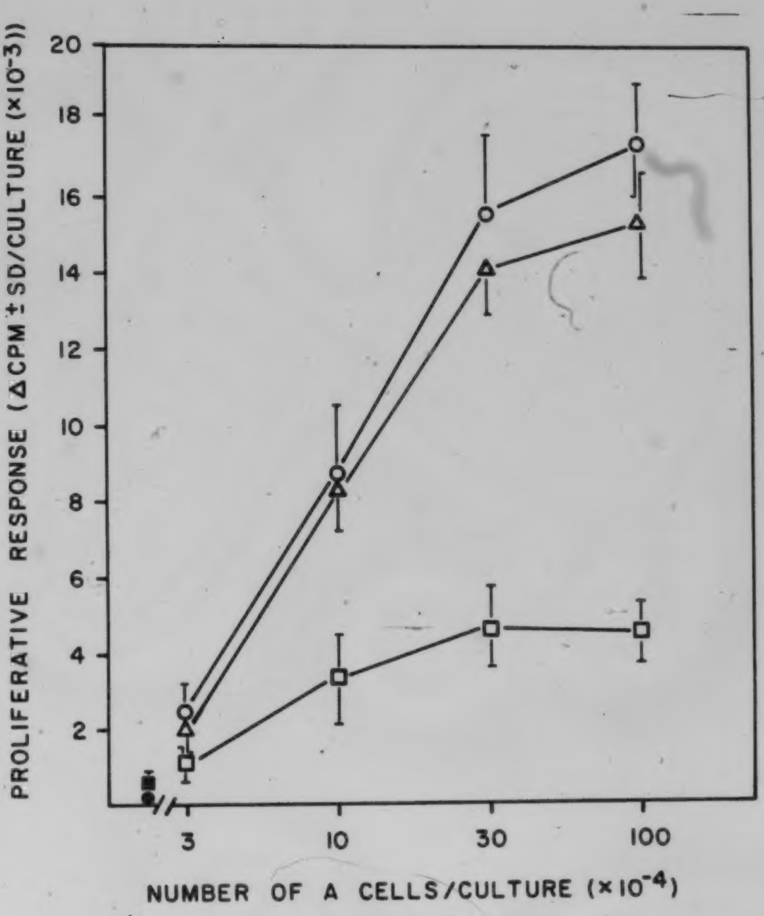


Figure 18. Specific inhibition of A cell dependent T cell activation by monoclonal anti-I-A^k antibody. CKA-T$_h$ cells (10^4/culture) were cultured alone (closed square), or with 15 μg/ml KLH in the absence (closed circle) or presence (open symbols) of the indicated numbers of CBA/CaJ irradiated spleen cells (A cells). Monoclonal αI-A^d (open triangles) or αI-A^k (open squares) antibodies were added as culture supernatants (1/1000 final dilution) of the MKD6 and 10-2.16 B cell hybridomas respectively. Responses to A cells without antigen have been subtracted. Proliferative responses were assessed on day 3.

Ia^{-}, IL-1 secreting MØ-like tumor (Koren *et al*, 1975; Mizel *et al*, 1978b) and P388AD.2, an Ia^{+}, IL-1 secreting DC-like tumor (Table 8) (Cohen and Kaplan, 1981; Cohen *et al*, 1982), both derived from the DBA/2 P388 murine leukemia, were as effective as DBA/2 PAC in restoring the response by NA spleen cells to TNP-Ba and TNP-ficoll (Figure 19). However, P388NA.10, an Ia^{+}, IL-1 nonsecreting tumor also derived from the P388 leukemia (Cohen and Kaplan, 1981; Cohen *et al*, 1982), was devoid of A cell activity in this system (Figure 19). We have confirmed the lack of Ia expression by P388D$_1$ and determined the percentage of Ia^{+} cells in P388AD.2, P388NA.10, and PAC by flow cytometry analysis of cells stained by indirect immunofluorescence (Figure 20).

IL-1, which is derived predominantly from MØ-like A cells (reviewed in Dinarello, 1984; Durum *et al*, 1985), is a critical lymphokine for antigen and APC dependent T cell activation (reviewed in Mizel, 1982). The inability of IL-1 nonsecreting P388NA.10 tumor cells (Figure 19) and glutaraldehyde fixed or UV treated SAC to reconstitute AFC responses to TI antigens in A cell deficient spleen cells (Figure 16) suggested a critical role for IL-1 secretion in TI B cell activation as well. This was directly confirmed by the observation that pure recombinant murine IL-1 completely restored the response of NA spleen cells to all four TI antigens tested (Figure 21). The effectiveness of IL-1 was not diminished by stringent T cell depletion (Table 9), suggesting a direct effect of IL-1 on B cells. IL-1 also restored immunocompetence in NA spleen cells from C3H/HeJ mice (LPS nonresponders), ruling out the possibility that the recombinant IL-1, which was provided as a bacterial cell lysate, contained LPS which accounted for its effect (data not shown).

D. Discussion

The A cell dependence of TI-2 B cell responses is well established (Chused *et al*, 1976; Lee *et al*, 1976; Pierce and Kapp, 1976a; Persson *et al*, 1977; Nordin, 1978; Boswell *et al*, 1980a,c; Letvin *et al*, 1981; Morrissey *et al*, 1981; Fernandez and Palacios, 1982, Mond, 1982). However, AFC responses to some TI-1 antigens have been reported to be either A cell dependent (Ishizaka *et al*, 1977; Haeffner-Cavaillon *et al*, 1982; Melchers *et al*, 1982; Bandeira

TABLE 8

Characteristics of DC-Like and M0-Like Cell Lines Used in These Studies

Characteristic[a]	Cell Line		
	$P388D_1$	P388AD.2	P388NA.10
Ia expression	–	+	+
IL-1 secretion	+	+	–
FcR	+	–	–
Phagocytosis	+	–	–
MLR stimulation	–	+	–
Ag presentation	–	+	–

[a] References: Koren *et al*, 1975; Cohen and Caplan, 1981; Cohen *et al*, 1982.

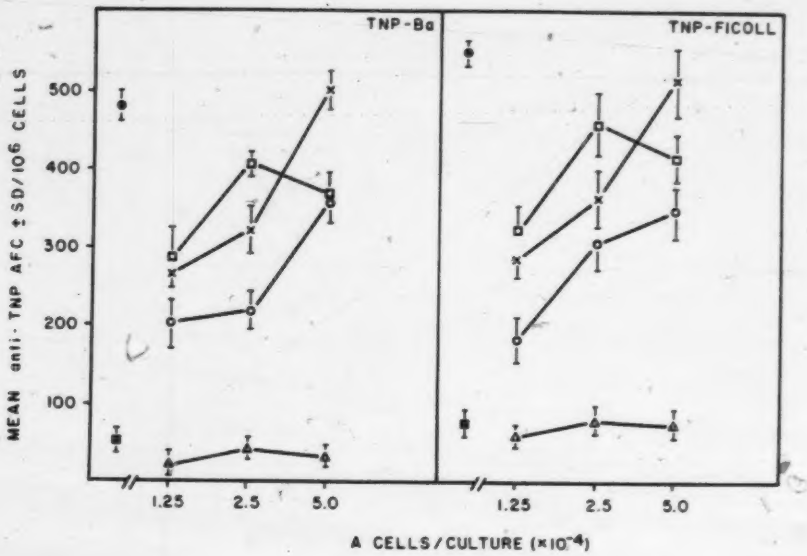


Figure 19. Function of A cell tumor lines in AFC responses to TI-1 and TI-2 antigens. CBA/CaJ normal (closed circles), NA (closed squares), or NA spleen cells reconstituted with DBA/2 PAC (x), P388AD.2 (open squares), P388D$_1$ (open circles), or P388NA.10 (open triangles), were cultured with TNP-Ba or TNP-ficoll.

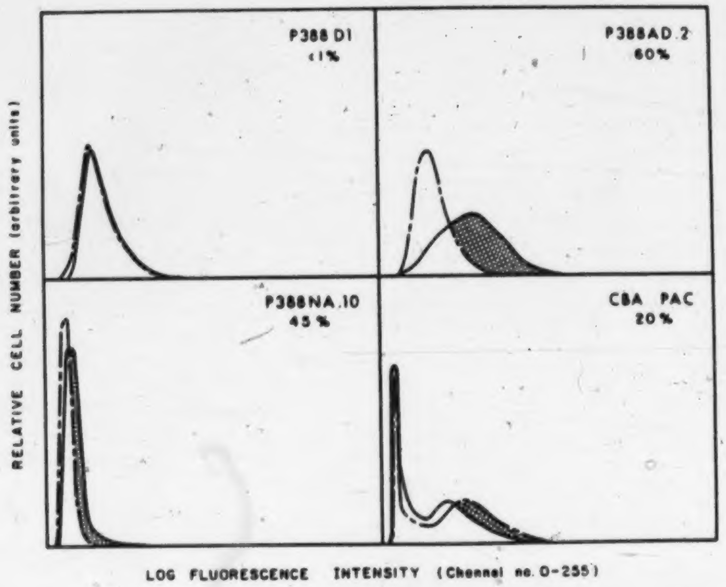


Figure 20. Flow cytometric analysis of Ia on A cells. Expression of Ia antigens on various A cells was quantitated by indirect immunofluorescence staining with 10-2.16 (I-A^k specific, broken lines) or MKD6 (I-A^d specific, solid lines) supernatants followed by flow cytometry analysis. The percentage of cells staining with intensities above that of the specificity control is indicated by shading and in the top right hand corner of each panel.

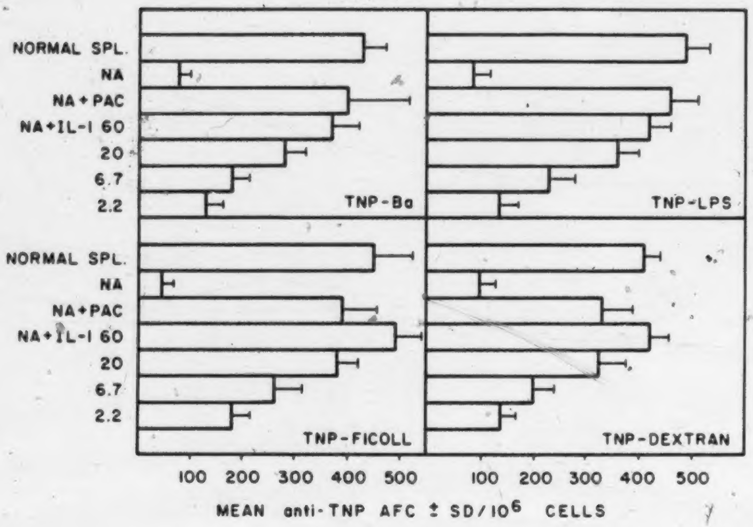


Figure 21. Recombinant interleukin-1 replaces A cell function. CBA/CaJ normal or NA spleen cells were cultured with TNP-Ba, TNP-LPS, TNP-ficoll, or TNP-dextran. NA spleen cells were reconstituted with 10^5 CBA/CaJ PAC or various concentrations (U/ml) of recombinant murine IL-1. Note: NA spleen cells cultured with IL-1 (60 U/ml) in the absence of antigen yielded no more AFC/10^6 cells than NA spleen cells alone without IL-1 or antigen.

TABLE 9

IL-1 Restores Responsiveness in T Cell Depleted, A Cell Deficient Spleen Cells

Spleen Cells	PAC	IL-1	Mean anti-TNP AFC ± SD/10^6 cells	
			C'	T cell depleted
Normal	–	–	523 ± 50	278 ± 47
NA	–	–	96 ± 8	62 ± 7
NA	10^5	–	244 ± 22	255 ± 39
NA	–	60 U/ml	266 ± 14	254 ± 32

CBA/CaJ spleen cells were treated with C' or αThy-1.2 and αLy-2.1 antibodies and C' (T cell depleted), after which they were depleted of A cells (NA) or left unfractionated (normal). NA spleen cells were reconstituted with CBA/CaJ PAC or recombinant IL-1. Cultures were challenged with TNP-Ba.

et al, 1983; Corbel and Melchers, 1983, 1984; Fernandez and Severinson, 1983; Diner *et al*, 1986) or A cell independent (Yoshinaga *et al*, 1972; Lemke *et al*, 1975; Chused *et al*, 1976; Lipsky and Rosenthal, 1976; Rosenstreich and Oppenheim, 1976; Kurland *et al*, 1977; Wong and Herscowitz, 1979; Boswell *et al*, 1980a,b). The studies reported here clearly demonstrate the A cell dependence of these responses; but, in agreement with Corbel and Melchers (1984), we find that rigorous A cell depletion methods must be employed to demonstrate this fact.

Our demonstration that IL-1 secretion by A cells is both necessary and sufficient for TI B cell activation may explain the lack of A cell dependence observed by some investigators in responses to some TI antigens. Thus, the reported A cell independence of AFC responses to LPS, Ba and other microbial products may be ascribed to the activation of IL-1 secretion by these agents (reviewed in Dinarello, 1984). Indeed, LPS stimulation of MØ is used for the preparation of IL-1 rich MØ supernatants (Unanue & Kiely, 1977; Mizel *et al*, 1978a). Activation of residual MØ is probably most effective when high LPS concentrations are used, since polyclonal B cell activation by mitogenic (high) concentrations of LPS was reported to be less A cell dependent than activation of specific B cells by lower concentrations of TNP-LPS (Martinez-Alonso *et al*, 1980). Complicating matters further are reports that B cells secrete IL-1 (Scala *et al*, 1984; Gerrard and Volkman, 1985; Matsushima *et al*, 1985) or express it in membrane bound form (Kurt-Jones *et al*, 1985) under certain activation conditions, including LPS stimulation. Finally, the use of fetal calf serum may obscure IL-1 dependence (Hoffmann *et al*, 1984). Clearly, however, any activity of B cell derived IL-1 was not sufficient for TI B cell activation under the serum free culture conditions used in our studies.

Boswell *et al* (1980a,b) failed to demonstrate A cell dependence of AFC responses to TNP-Ba and TNP-LPS. Furthermore, they reported that B cells are heterogeneous with respect to their requirement for A cells when activated by TI-1 antigens. Thus, the Lyb-5^+ B cell subset could be activated by TNP-Ba pulsed A cells, whereas the Lyb-5^- subset only responded to this antigen when present in free suspension. Lyb-5^- B cells appeared unable to receive activation signals from A cells. If activation of the Lyb-5^+ subset by TI-2 antigens is absolutely dependent upon A cell derived activation signals, then the immune defect in CBA/N mice, which lack this subset and fail to respond to TI-2 antigens (reviewed in Scher, 1982), could be due to the absence of effective A cell-B cell interactions in the Lyb-5^- subset. Since A cells from CBA/N mice present antigens normally (Boswell *et al*, 1980b), except for those under Ir gene control of the Ia.W39 specificity (Rossenwasser and Huber, 1981), the defect must reside in the B cells.

Contrary to the results of Boswell *et al* (1980a,b), we have found AFC responses (in spleen cells containing Lyb-5^- and Lyb-5^+ B cells) to TNP-LPS and TNP-Ba to be strictly A cell dependent (Figure 13). This discrepancy may be explained by postulating that rather than being A cell independent, the Lyb-5^- subset is merely less A cell (or IL-1) dependent than the Lyb-5^+ subset. Thus, A cell dependence of TI-1 AFC responses in Lyb-5^- or normal adult B cell populations, which contain approximately equal numbers of both subsets (Ahmed *et al*, 1977), would only be seen with stringent A cell depletion techniques, such as those used in our studies.

Our failure to demonstrate MHC restriction in TI AFC responses is difficult to reconcile with the observations of Singer and Hodes (1982) and Hodes *et al* (1983a,b) that B cells must recognize self Ia for TD and TI responses to TNP-ficoll. Although MHC restricted antibody has been reported (van Leewen *et al*, 1979; Wylie *et al*, 1982), the concomitant recognition of antigen plus Ia by B cell Ig receptors is not a requirement for B cell activation. Thus, until such time as the physiological relevance of A cell-B cell MHC restriction becomes understood as it is for T cell activation, the possibility that this interesting phenomenon may be due to experimental artefacts must be considered.

The ability of predominantly Ia$^-$ PAC (Figure 20) (Cowing *et al*, 1978) and the Ia$^-$ MØ-like tumor P388D$_1$ to provide A cell function in both TI-1 and TI-2 B cell responses apparently contradicts previous reports that Ia$^+$ SAC were required, possibly to process and present TI antigens for B cell activation (Letvin *et al*, 1981; Morrissey *et al*, 1981). However, the majority of splenic A cells express Ia antigens (Cowing *et al*, 1978); thus, these earlier studies did not directly assess the ability of Ia$^-$ A cells to function in TI B cell responses. Furthermore, antigen processing and MHC restricted antigen presentation were not investigated. We show that neither of these A cell functions is necessary for TI B cell activation (Figures 16 and 17; Table 7). Thus, the functions required of A cells clearly differ in T cell *vs* TI B cell activation.

In further contrast to T cell activation, functional heterogeneity of A cell subpopulations active in TI B cell activation was not observed. All A cell types capable of IL-1 secretion functioned adequately. Although SAC were more efficient than PAC on a per cell basis (Figure 14), this is likely explained by the low percentage of MØ in populations of resident peritoneal cells (Lee and Berry, 1977) as compared to SAC which are enriched for MØ and DC. Furthermore, large BM-MØ, which function poorly in T proliferative responses (Lee and Wong, 1980, 1982; Lee and Guidos, 1984), were considerably active in the TI B cell response to TNP-Ba (Figure 15). Activation of BM-MØ, which increases Ia expression and greatly enhances the accessory function of these cells in T cell activation (Lee and Wong, 1982), had little effect, other than increasing nonspecific suppressive activity at high MØ concentrations, on their function in the B cell response to TNP-Ba (Figure 15). Thus, a greater variety of A cell types are capable of participating in TI B cell activation than are functional in T proliferative responses.

Insofar as B cell activation by so-called TI antigens and mitogens is not truly T cell independent (Mond *et al*, 1980, 1983; Letvin *et al*, 1981; Jaworski *et al*, 1982; Zubler and Glasebrook, 1982; Endres *et al*, 1983; Nossal and Pike, 1984; Pike and Nossal, 1984; Pike *et al*, 1984), the question of how the necessary T_h are activated must be raised. In view of the lack of effect of A cell specific anti-Ia antibody on the AFC responses to TNP-Ba (Table 7), it is unlikely that Ia restricted A cell-T_h interactions are required. There is evidence, however, that antigen processing and MHC restricted antigen presentation by B cells (LoCascio *et al*, 1984; Lanzevecchia, 1985) may be important for the generation of nonspecific T cell derived B cell growth and differentiation factors. Alternatively, T cells may be activated to produce such factors in the absence of antigen *via* direct interaction with activated B cells (DeKruyff *et al*, 1985).

The results reported in this chapter suggest that the secretion of IL-1 is an essential A cell function in TI B cell activation. Moreover, the complete restoration of AFC responses in A cell depleted spleen cells by pure recombinant IL-1 suggests that no other A cell derived factors are required, contrary to reports by Corbel and Melchers (1984) that several species of A cell derived (α) factors could be distinguished. However, IL-1 does display molecular heterogeneity and exists in high molecular weight (35K), low molecular weight (17K), and very low molecular weight (2K and 4K) forms (reviewed in Durum *et al*, 1985). Furthermore, two related but distinct genes, termed α and β, encoding IL-1-like polypeptides have been cloned in both the human (Auron *et al*, 1985; March *et al*, 1985; van Damme *et al*, 1985) and murine (Lomedico *et al*, 1984) systems. The interrelationships of these molecules is not clear; whether they also display functional heterogeneity remains to be determined.

The failure of T cell depletion to abrogate the ability of IL-1 to restore AFC responses to TNP-Ba by A cell deficient spleen cells (Table 9) is consistent with a direct role for IL-1 in TI B cell activation. This idea is not without precedent in the immunological literature (Howard and Paul, 1983; Howard *et al*, 1983b; Giri *et al*, 1984; Pike and Nossal, 1985a). Although, in agreement with previous results (Endres *et al*, 1983; Mond *et al*, 1983; Pike and Nossal, 1984), the TNP-Ba response in unfractionated spleen cells was reduced somewhat by T cell depletion, IL-1 reconstituted normal NA and T cell depleted NA spleen cells to exactly the same extent (Table 9). Possibly, the IL-1 responsive and T cell factor responsive cells are members of nonoverlapping B cell subsets. Consistent with this, Pike and Nossal (1985a) have demonstrated that IL-1 supports the growth and differentiation of a proportion of FLU specific B cells cultured with FLU-ficoll in the absence of filler cells or additional cytokines. Moreover, an additive rather than a synergistic effect was observed upon the addition of both IL-1 and IL-2 to single FLU specific B cells. Thus, B cells may be heterogeneous with respect to their requirement for various A cell or T cell derived cytokines.

In summary, we have demonstrated that A cell functions differ considerably in T cell activation and TI B cell activation. In the latter process, neither antigen processing, MHC restricted antigen presentation, nor expression of Ia antigens by A cells is required; A cell secretion of IL-1 is both necessary and sufficient to allow B cell activation by TI antigens in A cell deficient spleen cells. The data are consistent with the notion that IL-1 acts directly on B cells; however, the point(s) in the activation cascade at which IL-1 acts remain to be determined. Studies designed to answer this question are currently in progress.

VI. Mechanism of TNP-CMC/MC Induced, Accessory Cell Dependent B Cell Tolerance

A. Introduction

The importance of MØ and other adherent accessory cells in T cell activation and TI B cell activation is well established (reviewed in Möller and Coutinho, 1975; Pierce and Kapp, 1976a; Rosenstreich and Oppenheim, 1976; Steinman, 1981; Howard and Paul, 1983; Unanue, 1984; Schwartz, 1985). Recent reports, and data presented in ***Chapter IV*** of this thesis, also ascribe a pivotal role to A cells in the induction of B cell tolerance (Diener *et al*, 1976; Phipps and Scott, 1983; Goldings, 1986a,b). Moreover, MØ are required to convert T cell derived, antigen nonspecific, SIRS to the biologically active form (Tadakuma and Pierce, 1976), possibly by an oxidative process (Aune and Pierce, 1981c). MØ also exert cytostatic and cytotoxic effects on other cell types by oxidative mechanisms (reviewed in Adams and Nathan, 1983; Adams and Hamilton, 1984). However, the nonspecific nature of both positive and negative (largely prostaglandin mediated) immunoregulation by A cells is difficult to reconcile with their antigen specific role in the induction of tolerance by hapten-CMC/MC conjugates (***Chapter IV***). Although the importance of A cell derived IL-1 in TI B cell activation was demonstrated in ***Chapter V*** of this thesis, the possibility that CMC/MC treatment of A cells renders them incapable of IL-1 secretion or enhances prostaglandin secretion would not seem to explain the hapten specificity of CMC/MC induced, A cell dependent tolerance. However, in view of recent studies implicating A cell derived IL-1, prostaglandins, and H_2O_2 in hapten specific, Fc dependent tolerance induced by TNP-HGG (Goldings, 1986a,b), this possibility was directly assessed. Furthermore, we investigated the possibility that tolerizing A cells exert their effect through release at close range of peroxide or peroxide-like substances. Finally, the relationship of CMC/MC induced, A cell dependent tolerance to existing models of B cell tolerance was examined.

B. Materials and Methods

Antigens. FLU-POL was prepared following the methods of Goding (1976) and Nossal *et al* (1978). Briefly, POL (prepared from *Salmonella* strain 1699, Ada *et al*, 1964) was dialyzed overnight against Na_2CO_3 buffer (0.2 M, pH 9.3). The following day, 0.2 ml FITC (1 mg/ml, isomer 1, Sigma Chemical Co., St. Louis, MO) dissolved in dimethyl sulfoxide was added dropwise to 1 ml (4 mg/ml) of dialyzed POL, with constant stirring. The solution was allowed to stand 2 hr at room temperature in the dark, then dialyzed extensively against PBS. The hapten substitution ratio was determined as the molarity of the hapten divided by the molarity of POL. FLU concentration was determined by absorbance at 495 nm ($\epsilon = 60{,}000$). POL was converted to monomeric POL (MON) (MW 40,000) by treatment with 0.05 N HCl for 20 min prior to measurement of absorbance at 215 nm ($\epsilon = 9.4$). The substitution ratio of FLU-POL used in these studies was 1.

Flow Cytometry Analysis of FLU Binding, mIg$^+$ Spleen Cells. Spleen cells (10^6/well) in V-bottom microtiter wells were pretreated for 30 min on ice with HBSS containing 0.2% gelatin and 0.1% azide to block nonspecific binding. POL (500 μg/ml) was included in those groups to be stained with FLU-POL. The medium was then replaced with 0.1 ml HBSS containing 0.1% gelatin, 0.1% azide, and (1 μg/ml) FLU-POL or biotin conjugated $F(ab')_2$ fragments of goat anti-mouse Ig (G+M+A, Cappel, Cochraneville, PA) diluted 50 times. After 15 min on ice, cells were washed 3 times and the FLU-POL stained groups were fixed with 2% formaldehyde-PBS. The anti-Ig stained cells were incubated for 15 min on ice with avidin-phycoerythrin (Becton Dickinson, Mountain View, CA) diluted 20 times in HBSS containing 0.1% gelatin and 0.1% azide. Cells were then washed 3 times and fixed in 2% formaldehyde-PBS prior to analysis. Cells to be subjected to two-color flow cytometry analysis were stained first with FLU-POL, followed by biotin-anti-Ig, and finally avidin-phycoerythrin. Flow cytometry analysis of FLU binding cells (green fluorescence) was performed on an EPICS V FACS as described in *Chapter V*. Analysis of mIg$^+$ cells (red fluorescence) was performed using an argon laser exciting at 488 nm, a 515 nm blocking filter, 560 nm dichroic filter, and a 570 nm longpass filter. For two-color analysis, a 530 nm shortpass filter was also included. Data were collected gated on forward angle light scatter and red fluorescence or green fluorescence. The data shown in Figure 26 were collected gated on forward angle light scatter and red fluorescence, but were no different than data collected gated on forward angle light scatter and green fluorescence (data not shown). Thus, no cells were detected exhibiting green fluorescence in the abscence of red fluorescence. Spleen cells from individual mice (3 mice/group) were analyzed separately. Similar results were obtained from mice treated and analyzed on two separate occasions. Representative histograms are shown.

Antibody Forming Cell Assay Following In Vivo Challenge. Mice were killed by cervical dislocation and their spleens removed into 5 ml MDBSS. Cell suspensions were prepared from individual spleens and debris removed by sedimentation at 1 *g* for 7 min. After washing, cells were resuspended in 8 ml MDBSS and 0.2 ml were admixed with 0.085 ml MDBSS, 0.02 ml guinea pig complement diluted 3 times in MDBSS, and 0.025 ml haptenated SRBC target (10% v/v, prepared as described in *Chapter III*). Following thorough mixing, 0.05 ml of this mixture was placed in a double-walled slide chamber, after which the procedure was identical to that described in *Chapter III* for *Antibody Forming Cell Assay Following In Vitro Challenge.*

Other materials and methods employed for these studies have been fully described in *Chapters III, IV and V.*

C. Results

Failure of Indomethacin or IL-1 to Restore Immunocompetence in TNP-MC Treated Spleen Cells

The most intriguing aspect of our observation is that the A cell deficiency in CMC/MC tolerant mice is hapten specific (*Chapter IV*). A cells release both positive (e.g., IL-1, reviewed in Durum *et al*, 1985) and negative (e.g., prostaglandins, reviewed in Adams and Nathan, 1983) immunoregulatory molecules which are known to function in an antigen nonspecific manner. Thus, the possibility that TNP tolerizing A cells are defective in IL-1 release is unlikely to explain the observation that the same A cells are fully competent in supporting a response to FLU-Ba. Similarly, release of inhibitory prostaglandins by tolerizing

A cells should not account for the hapten specific defect. As expected, neither IL-1 nor indomethacin (an inhibitor of prostaglandin synthesis) (Adams and Nathans, 1983), were capable of restoring immunocompetence in TNP-MC treated spleen cells (Table 10).

A Cell Dependent Tolerance is Not Mediated by a Soluble Factor

The hapten specificity of A cell dependent tolerance could be a result of the hapten being displayed on the A cell surface *via* surface bound carrier. Alternatively, A cells could convert TNP-CMC/MC into suppressive molecules by an oxidative process, as is the case for T cell derived antigen nonspecific SIRS (Aune and Pierce, 1981c). In the second instance, hapten specifically suppressive molecules should be detectable in supernatants of TNP-CMC/MC treated A cells. To distinguish between these two possibilities, supernatants from TNP-MC treated PAC were collected and assayed for the ability to induce TNP specific tolerance. The results show that supernatants from normal or TNP-MC treated PAC reconstituted NA spleen cells to the same extent (Figure 22). Thus, there is no evidence for the release of specific suppressive molecules by A cells in this system.

There are at least two possible explanations of the requirement for close cellular interactions in A cell dependent CMC/MC mediated tolerance. First, the A cells could function passively to present locally high concentrations of antigen in a multivalent array, thus favoring tolerance induction (Feldmann and Diener, 1972). Such a mechanism likely accounts for the greater efficiency of Fc dependent tolerance induction by hapten-SGG conjugates in the presence of FcR^+ A cells (Phipps and Scott, 1983; Phipps *et al*, 1984). However, the failure of NA cells to be rendered tolerant in the absence of A cells, even with several-fold higher TNP-MC concentrations (Table 11), makes it unlikely that A cells are merely serving to present tolerogen in a more concentrated fashion in this system. The alternative explanation is that A cells are actively involved in CMC/MC induced tolerance. Consistent with this, glutaraldehyde fixed PAC from TNP-CMC tolerant mice failed to induce TNP specific B cell tolerance (Table 12). The mode of cell-cell interaction in this system does not require recognition of Ia molecules, since monoclonal anti-I-A^k antibody failed to prevent tolerance induction by

TABLE 10

Failure of Indomethacin or IL-1 to Restore Immunocompetence in TNP-MC Treated Spleen Cells

Group	Indomethacin	IL-1	Mean anti-TNP AFC ± SD/10^6 cells
A	–	–	360 ± 28
	3 μg/ml	–	340 ± 32
	–	60 U/ml	372 ± 41
B	–	–	36 ± 10
	3 μg/ml	–	47 ± 8
	–	60 U/ml	25 ± 9

CBA/CaJ spleen cells were treated with RPMI containing 10% FCS alone (Group A) or containing 100 μg/ml TNP_4MC (Group B) for 90 min at 4° C. Cells were then washed 3 times prior to challenge with TNP-Ba. Indomethacin or recombinant IL-1 were added to the indicated groups at the time of challenge.

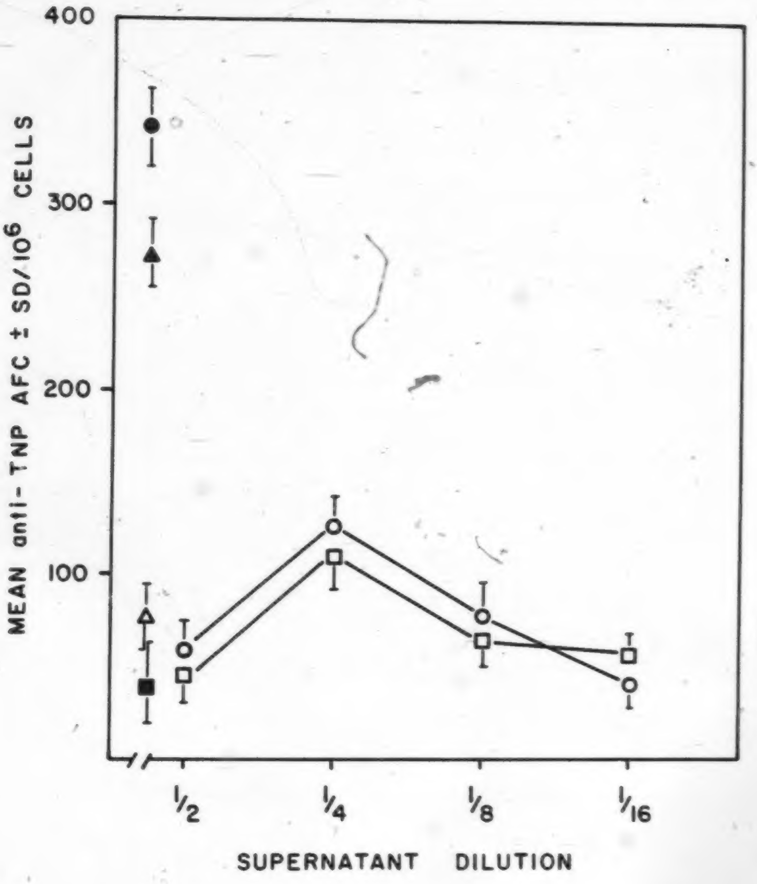


Figure 22. Failure of supernatants from TNP-MC treated A cells to induce tolerance in A cell deficient spleen cells. CBA/CaJ normal (closed circle) or NA (closed square and open symbols) spleen cells were cultured with TNP-Ba. NA spleen cells were reconstituted with 10^5 CBA/CaJ normal PAC (closed triangle), TNP-MC treated PAC (100 μg/ml for 90 min at 4° C, open triangle), or supernatants from normal (open circles) or TNP-MC treated (open squares) PAC at the indicated dilutions. To obtain supernatants, 2 x 10^6 normal or TNP-MC treated PAC were cultured in 2 ml RPMI combining 10% FCS in 24-well Linbro trays at 37° C. After 18 hr, supernatants were collected, centrifuged and filtered to remove nonadherent peritoneal cells, and used to reconstitute NA cells as indicated.

TABLE 11

Failure of TNP-MC to Induce Tolerance *In Vitro* in the Absence of A Cells

Spleen Cells	TNP-MC	PAC	Mean Anti-TNP AFC ± SD/10^6 cells
Normal	-	-	289 ± 43
Normal	100 μg/ml	-	15 ± 8
NA	-	-	8 ± 5
NA	-	+	209 ± 31
NA	50 μg/ml	+	201 ± 18
NA	100 μg/ml	+	216 ± 25
NA	200 μg/ml	+	185 ± 33
NA	300 μg/ml	+	219 ± 27
NA	400 μg/ml	+	176 ± 28

Normal or NA CBA/CaJ spleen cells were treated with RPMI containing 10% FCS alone or containing the indicated concentrations of TNP_4MC for 90 min at 4° C. Cells were then washed 3 times and cultured with TNP-Ba. Where indicated, NA spleen cells were reconstituted with 10^5 CBA/CaJ PAC.

TABLE 12

Glutaraldehyde Fixation of A Cells from Tolerant Mice Abrogates A Cell Dependent Tolerance Induction

Spleen Cells[a]	PAC[b]		IL-1[c]	Mean anti-TNP AFC ± SD /10^6cells
	Type	Glutaraldehyde		
Normal	-	-	-	198 ± 32
NA	-	-	-	22 ± 8
NA	normal	-	-	139 ± 25
NA	normal	+	-	14 ± 10
NA	normal	+	+	122 ± 16
NA	tolerant	-	-	22 ± 12
NA	tolerant	-	+	27 ± 11
NA	tolerant	+	+	97 ± 21

[a] CBA/CaJ normal or NA spleen cells were challenged with TNP-Ba.

[b] NA spleen cells were reconstituted with 10^5 CBA/CaJ PAC from normal mice, or mice that had been injected with 300 μg TNP_6CMC 48 hr previously (tolerant). Where indicated, PAC were glutaraldehyde fixed for 30 s as described in Table 6.

[c] Where indicated, IL-1 (60 U/ml) was included in culture.

CBA/CaJ ($H\text{-}2^k$) PAC from TNP-CMC tolerant mice (Table 13).

The Reducing Agent KI Reverses the Tolerance Inducing Capability of A Cells From TNP-CMC Treated Mice.

The preceeding experiments suggest that the induction of tolerance by A cells is an active process. Possibly, suppressive MØ are activated by the tolerogen. Such MØ may exert their cytostatic or cytotoxic effect on other cells through superoxides or peroxide-like substances (Aune and Pierce, 1981c; Adams and Nathans, 1983). To test this hypothesis, peritoneal A cells from TNP tolerant mice were incubated for 30 min at 4° C with 6 x 10^{-4} M KI as a reducing agent in the expectation that it may neutralize MØ derived oxidants (Aune and Pierce, 1981c). These cells were then washed 3 times and used to reconstitute immunocompetence in cultures of NA spleen cells. Incubation of A cells from TNP-CMC treated mice with KI indeed abolished their "tolerant" state, completely restoring their capacity to reconstitute responsiveness in NA spleen cells (Table 14). We conclude from this experiment that oxidative processes are associated with and might possibly be responsible for "tolerance" in A cells.

The effect of KI on unfractionated spleen cells from tolerant mice was also evaluated. Treatment of such cells with KI within the critical period of 24 to 42 hr after the *in vivo* administration of the tolerogen resulted in partial but significant reversal of the state of unresponsiveness (Table 15). The ability of KI to partially reverse tolerance 24 or 42 hr after the injection of the tolerogen suggests that at this time point, some B cells have become irreversibly tolerant *in vivo*. This interpretation is consistent with data referred to in *Chapter IV* (Table 5), indicating that given sufficient time, TNP-MC treated A cells irreversibly and hapten specifically compromise the immunocompetence of B cells.

TABLE 13

Monoclonal αI-A^k Antibody Does Not Prevent Induction of A Cell Mediated B Cell Tolerance

Spleen Cells	PAC	αI-A^k	Mean Anti-TNP AFC ± SD/10^6 cells
Normal	–	–	287 ± 38
NA	–	–	29 ± 17
NA	normal	–	223 ± 27
NA	normal	+	238 ± 31
NA	tolerant	–	42 ± 12
NA	tolerant	+	47 ± 18

CBA/CaJ normal or NA spleen cells were challenged with TNP-Ba. Where indicated, NA spleen cells were reconstituted in the presence or absence of 10-2.16 (αI-A^k) culture supernatant (1/1000) with 10^5 CBA/CaJ PAC from normal mice or mice that had been injected 48 hr previously with 300 μg TNP_6CMC.

TABLE 14

The Reducing Agent KI Reverses "Tolerance" in A Cells from Mice Treated with the Tolerogen TNP-MC

Spleen Cells	Reconstituted with				Mean anti-TNP AFC ± SD/10^6 cells
	normal A cells	normal A cells (KI)[a]	tolerant A cells	tolerant A cells (KI)[a,b]	
Normal	–	–	–	–	270 ± 57
Tolerant[b]	–	–	–	–	33 ± 15
NA	–	–	–	–	19 ± 15
NA	+	–	–	–	178 ± 19
NA	–	–	+	–	47 ± 13
NA	–	+	–	–	173 ± 59
NA	–	–	–	+	188 ± 32

[a] A cells from normal or tolerant mice were incubated for 2 hr at 4° C with 6 x 10^{-4} M KI, washed 3 times and used for reconstitution (2 x 10^5/culture).

[b] Tolerant spleen cells or A cells were obtained from mice injected with 300 μg of TNP-CMC 48 hr prior to use. Cultures were challenged with TNP-Ba.

TABLE 15

The Reducing Agent KI Partially Reverses Tolerance in Unfractionated Spleen Cells

Spleen Cells	KI treatment[b]	Mean anti-TNP AFC ± SD/10^6 cells
Normal	-	328 ± 30
Normal	+	262 ± 19
Tolerant (24 hrs)[a]	-	13 ± 22
Tolerant (24 hrs)	+	111 ± 17
Tolerant (42 hrs)	-	<10
Tolerant (42 hrs)	+	79 ± 37

[a] Mice were rejected with 300 μg of TNP_6CMC and killed after 24 or 42 hr.

[b] Spleen cells were incubated with 1.2 x 10^{-6} M KI at 4° C for 30 min and then washed 3 times prior to culture. Cultures were challenged with TNP-Ba.

Relationship of Tolerance Induced by CMC/MC to Existing Models of B Cell Tolerance

The Two Signal Model

The data presented in this thesis and elsewhere (Diner *et al*, 1979; Diener *et al*, 1981; von Borstel *et al*, 1983) suggest that CMC/MC are novel tolerogens whose capacity to induce T cell independent, carrier and A cell dependent B cell unresponsiveness is not explained by existing models of B cell tolerance. The validity of this notion was assessed by several means.

One model of self-nonself discrimination by B cells, the two signal theory, (Brestcher and Cohn, 1970), proposes that the outcome of an antigenic encounter (Signal 1) by a B cell is determined by the presence or absence of appropriate "helper signals" (Signal 2) in the environment. This notion has gained support from observations demonstrating an inverse correlation between an antigen's tolerogenic and immunogenic capacities (Havas, 1969; Roelants and Goodman, 1970; Borel, 1971); Golan and Borel, 1974). Moreover, the presence of carrier specific T_h or Signal 2 replacing mitogens has been shown to interfere with hapten specific tolerance induction in some systems (Metcalf and Klinman, 1976; Nossal and Pike, 1979). Therefore, we tested the effect of T cell derived helper signals on the induction of A cell dependent, TNP-CMC mediated tolerance.

A KLH specific T_h cell line, capable of proliferating in an antigen specific manner (Table 16) and delivering antigen specific cognate help to antigen specific B cells (Figure 23) was generated according to Kimoto and Fathman (1980). It is important to note that TNP-MC treatment of splenic A cells did not impair the activation of CKA-T_h (Table 16). In contrast to other systems, neither IL-1 (Table 12, line 7), nor carrier specific help (Table 17) interfered with tolerance induction by TNP-MC treated A cells. This provides further evidence that CMC/MC represent novel tolerogens whose mode of action is significantly different from other tolerogens described to date.

Clonal Deletion/Abortion

Clonal deletion/abortion models of B cell tolerance propose that B cells are deleted during a tolerance sensitive phase of development of the animal or B cell, respectively.

TABLE 16

The CKA-T_h Line Proliferates in an Antigen Specific Manner

Antigen (concentration)	Proliferative Response[a] (mean cpm ± SD (x 10^{-3}))
None	4.7 ± 0.7
KLH (0.1 μg/ml)	29.6 ± 1.7
TNP-KLH (0.1 μg/ml)	28.2 ± 1.7
KLH (25 μg/ml)	36.9 ± 2.7
KLH (25 μg/ml)[b]	24.9 ± 3.2
OVA (0.1 μg/ml)	5.9 ± 0.7
OVA (25 μg/ml)	6.3 ± 1.4

[a] CKA-T_h cells (10^4/culture) were cultured with irradiated CBA/CaJ spleen cells (10^6/culture) in the presence of the antigens indicated. Proliferative responses were assessed on day 3.

[b] For this group, spleen cells were pretreated with TNP_4MC for 90 min at 4° C.

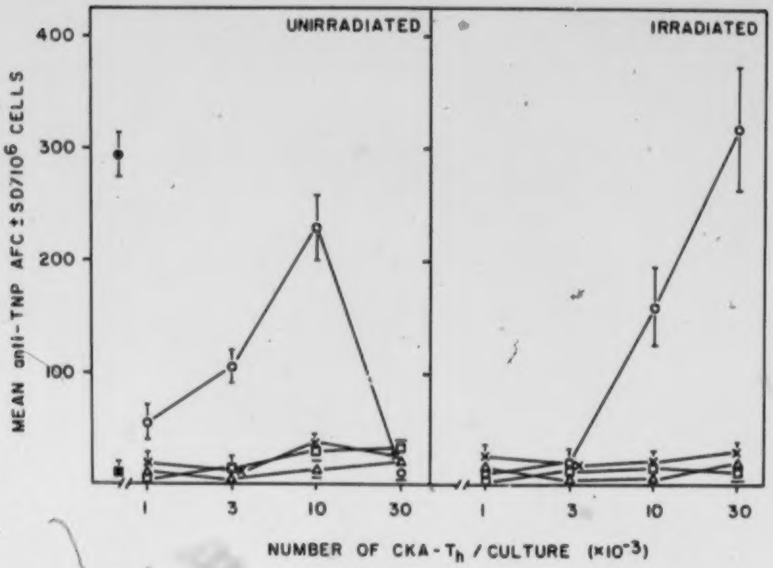


Figure 23. CKA-T_h cells deliver antigen specific cognate help to B cells. CBA/CaJ spleen cells (10^6/culture) were cultured with TNP-Ba (closed circle) or 0.1 μg/ml TNP-KLH (closed square). Unirradiated (left panel) or irradiated (right panel) CKA-T_h cells (open symbols) and 0.1 μg/ml TNP-KLH (open circles), KLH (open squares), TNP-OVA (open triangles) or TNP-OVA + KLH (x) were added as indicated. Culture conditions and assay are described in *Chapter III*.

TABLE 17

The Presence of Carrier Specific T_h Does Not Prevent Tolerance Induction by TNP-MC Treated A Cells

Spleen Cells[a]	Antigen	SAC	No. of CKA-T_h/Culture ($x\ 10^{-3}$)	Mean anti-TNP AFC ± SD /10^6 cells
Normal	TNP-KLH	–	–	25 ± 8
Normal	TNP-KLH	–	3	190 ± 25
Normal	TNP-KLH	–	10	324 ± 13
Normal	KLH	–	10	15 ± 6
NA	TNP-KLH	–	–	<10
NA	TNP-KLH	Normal	–	26 ± 7
NA	TNP-KLH	Normal	3	152 ± 18
NA	TNP-KLH	Normal	10	294 ± 35
NA	TNP-KLH	TNP-MC	3	12 ± 4
NA	TNP-KLH	TNP-MC	10	17 ± 8

[a] CBA/CaJ normal or NA spleen cells (10^6/culture) were cultured with 0.1 μg/ml TNP-KLH or KLH. Where indicated, 10^5 normal or TNP_4MC treated (100 μg/ml for 90 min at 4° C) SAC were added. The responses induced by TNP-Ba in NA cells reconstituted with normal or TNP_4MC treated SAC were 367 ± 41 and 21 ± 9 respectively.

[b] The KLH specific T cell line CKA-T_h was generated as described in *Chapter V*. The antigen specific helper function of this line for AFC responses is demonsrated in Figure 23.

However, in view of the existence of antigen binding B cells in animals tolerant to certain antigens (reviewed in Elson *et al*, 1977; Nossal, 1983), Nossal and Pike (1980) proposed the term "clonal anergy" to refer to clones which have been functionally silenced without being physically deleted. To differentiate between physical deletion and functional anergy in our system, the numbers of FLU binding B cells in normal and FLU tolerant mice were assessed by flow cytometry.

Initially, the fluorescence of FLU binding spleen cells from FLU-Ba immunized or FLU-CMC pretreated, FLU-Ba immunized mice were compared. The specificity of the FLU-POL staining was assessed by comparing the fluorescence profiles of spleen cells from unimmunized and FLU-Ba immunized mice (Figure 24A). Spleen cells from unimmunized mice contained small but detectable numbers of FLU binding cells, whereas spleen cells from FLU-Ba immunized mice contained significantly more FLU binding cells. Mice which had been treated with FLU-CMC 48 hr prior to immunization with FLU-Ba were hapten specifically tolerant, and consistent with this, no increase in FLU binding cells relative to unimmunized mice was detectable (Figure 24B). Thus, no clonal expansion of FLU binding cells occurred in FLU-CMC tolerant, FLU-Ba challenged mice. This effect was specific for the FLU-CMC tolerogen, since mice treated with the tolerogen TNP-CMC prior to FLU-Ba immunization contained numbers of FLU binding cells comparable to the FLU-Ba immunized control (Figure 24C).

To be certain that the FLU binding cells being detected were all B cells, samples were simultaneously stained with FLU-POL followed by a biotin-anti-Ig conjugate and avidin-phycoerythrin. Thus, two-color flow cytometry could be performed. Figure 25A demonstrates the specificity of the anti-Ig reagent. Spleen cells from normal mice contained 10% mIg$^+$ cells. This increased to 40% upon immunization with FLU-Ba. Moreover, the red fluorescence profile of anti-Ig-phycoerythrin stained spleen cells from FLU-Ba immunized mice and FLU-CMC pretreated, FLU-Ba immunized mice were virtually identical (Figure 25B). Thus, FLU-CMC treatment *in vivo* does not generally immunocompromise mice, further confirming the hapten specific B cell unresponsiveness in these mice.

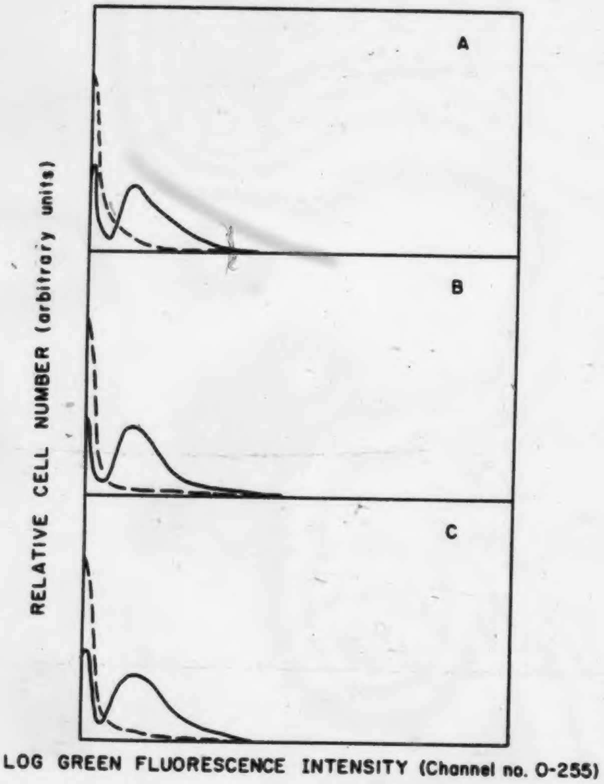


Figure 24. Flow cytometric analysis of FLU binding spleen cells from normal and FLU-CMC treated mice immunized with FLU-Ba. Where indicated, mice were injected i.v. with 300 μg FLU_5CMC or TNP_6CMC 48 hr prior to immunization i.v. with 4 x 10^{10} FLU-Ba organisms. After 7 days, FLU binding cells present in the spleens were analyzed by flow cytometry. The panels depict the fluorescence profiles of FLU binding spleen cells from mice that were (A) FLU-Ba immunized (solid line) *vs* normal unimmunized (broken line), (B) FLU-Ba immunized (solid line) *vs* FLU_5CMC treated prior to immunization with FLU-Ba (broken line), and (C) injected with TNP_6CMC (solid line) or FLU_5CMC (broken line) prior to immunization with FLU-Ba. The percentages of FLU binding cells, determined from channel by channel subtraction of the nonspecific fluorescence of unstained cells were as follows: FLU-Ba immunized, 8.6%; TNP_6CMC pretreated, FLU-Ba immunized, 7.8%; FLU_5CMC pretreated, FLU-Ba immunized, 0.11%; and normal unimmunized, 1.04%. The specificity of hapten specific tolerance and priming was confirmed by determining numbers of anti-FLU and anti-TNP AFC/spleen for each group.

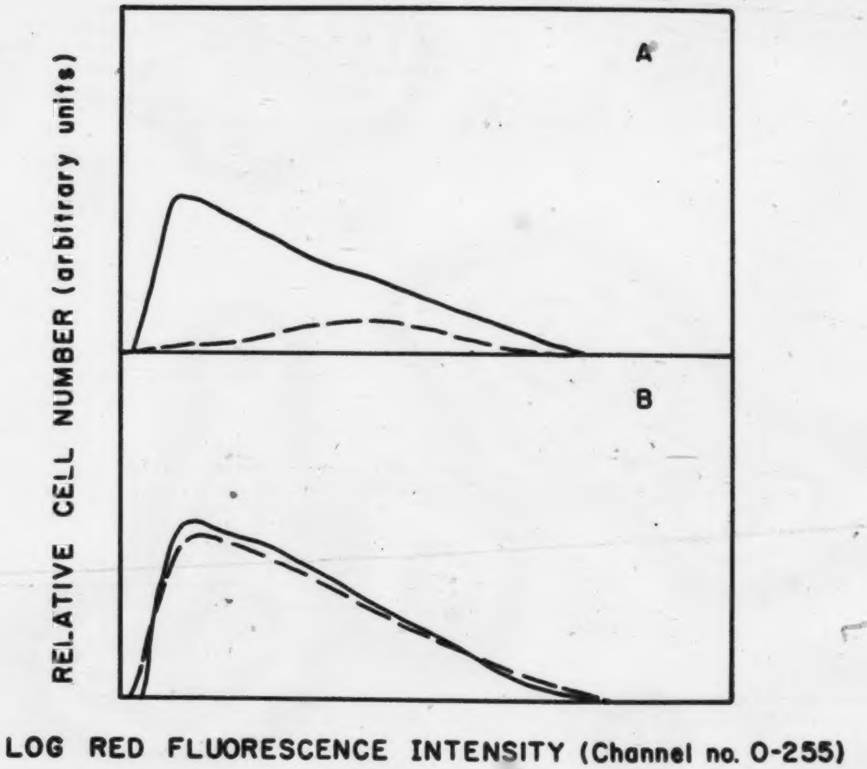


Figure 25. Flow cytometric analysis of mIg$^+$ spleen cells from normal, FLU-CMC tolerant and FLU-Ba immunized mice. Where indicated, mice were injected i.v. with 300 μg FLU$_5$CMC 48 hr prior to immunization i.v. with 4 x 10^{10} FLU-Ba organisms. The percentage of mIg$^+$ cells in the spleen was determined 7 days later by flow cytometry. The fluorescence profiles of mIg$^+$ spleen cells from mice that were (A) normal unimmunized (broken line) *vs* FLU-Ba immunized (solid line); and (B) FLU-Ba immunized (broken line) *vs* FLU$_5$CMC treated, FLU-Ba immunized (solid line), are depicted. The percentages of mIg$^+$ cells, determined from channel by channel subtraction of the nonspecific fluorescence of unstained cells were 10%, 40%, and 36% for normal (unprimed); FLU-Ba immunized; and FLU$_5$CMC pretreated, FLU-Ba immunized mice respectively.

The results of the two-color analysis are depicted in Figure 26. Spleen cells from unimmunized mice (Figure 26A) contained small, but reproducibly detectable numbers of mIg^+, FLU binding cells (dull green, bright red). No FLU binding, mIg^- cells were detected indicating that green fluorescence truly reflected FLU binding B cells. Spleen cells from mice immunized with FLU-Ba contained 3 populations of FLU binding, mIg^+ cells: dull green, bright red; medium green, dull red; and dull green, medium red (Figure 26B). These latter 2 populations likely reflect FLU primed B cells, perhaps actively secreting Ig. TNP-ficoll immunized (Figure 26C) or TNP-CMC tolerant mice (Figure 26D) both contained only the dull green, bright red population seen in the unimmunized control (Figure 26A). Finally, spleen cells from FLU-CMC treated mice contained barely detectable numbers of FLU binding, mIg^+ cells (Figure 26E). The percentage of FLU binding, mIg^+ cells in each group is shown in Table 18. While the percentages of FLU binding cells in spleen cells from unimmunized, TNP-ficoll immunized, or TNP-CMC treated mice were small, these numbers were reproducible, not statistically different, and similar to the numbers of FLU binding spleen cells reported by Nossal *et al* (1978). Interestingly, spleen cells from FLU-CMC treated mice contained approximately 10-fold fewer FLU binding, mIg^+ cells than normal and other control mice (TNP-ficoll immunized or TNP-CMC tolerant, Table 18). This difference is highly statistically significant ($p<0.005$). Thus, these results are consistent with an A cell dependent clonal deletion mechanism of CMC/MC induced B cell tolerance.

D. Discussion

Current concepts of A cell-B cell interaction do not provide an explanation for hapten specific unresponsiveness mediated by A cells. Goldings (1986a,b,) has recently demonstrated that MØ derived nonspecific immunoregulatory molecules, such as IL-1, prostaglandin E_2, and H_2O_2 function antagonistically in hapten specific, HGG mediated tolerance. Interestingly, the presence of indomethacin or catalase during the tolerance induction phase of the culture prevented tolerance, thus supporting a role for MØ derived prostaglandins and H_2O_2 in B cell tolerance. IL-1 also interfered with tolerance induction, but this protective effect was

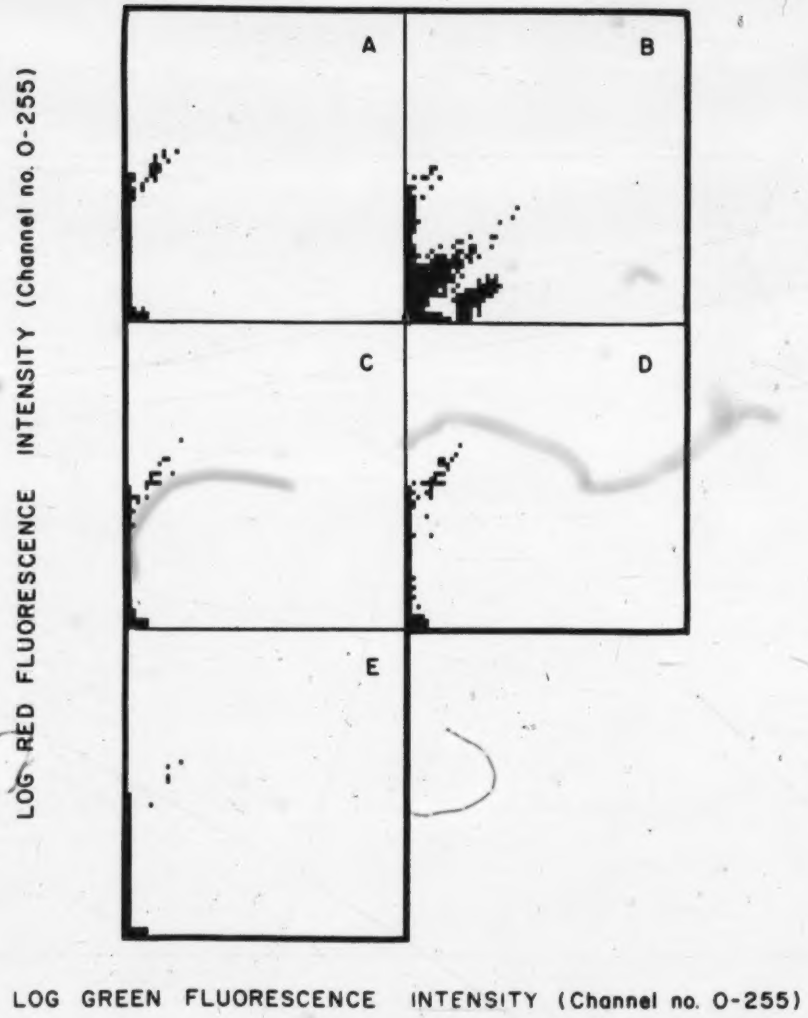


Figure 26. Two-color flow cytometric analysis of FLU binding, mIg$^+$ spleen cells from normal and FLU-CMC tolerant mice. Where indicated, mice were injected with 300 μg FLU$_5$CMC or TNP$_6$CMC or 4 x 10^{10} FLU-Ba organisms 7 days prior to FACS analysis. Results are depicted as a computer generated 2 parameter contour plot of log green fluorescence (FLU binding cells) *vs* log red fluorescence (mIg$^+$ cells). The numbers of cells per channel are represented as differences in shading. The red *vs* green fluorescence profiles of spleen cells from (A) normal (unimmunized), (B) FLU-Ba immunized, (C) TNP-ficoll immunized, (D) TNP$_6$CMC tolerant, and (E) FLU$_5$CMC tolerant are shown.

TABLE 18

Percentage of FLU Binding Spleen Cells in Normal, FLU-CMC Tolerant, and FLU-Ba Immunized Mice

Mice Treated with[a]		%FLU-binding, mIg$^+$ Cells[b] (mean ± SD)
Tolerogen	Antigen	
–	–	0.90 ± 0.19
–	TNP-ficoll	0.94 ± 0.22
TNP-CMC	–	0.85 ± 0.13
FLU-CMC	–	0.11 ± 0.02[c]

[a] CBA/CaJ mice (3 mice/group) were injected with 300 μg TNP_6CMC or FLU_5CMC, or 20 μg TNP-ficoll 7 days prior to two-color FACS analysis of FLU binding, mIg$^+$ cells.

[b] The percentage of FLU binding, mIg$^+$ cells was determined by subtraction of the background fluorescence of unstained cells for each sample.

[c] This value is significantly different ($p<0.005$, one-tailed *t* test,) from the three groups which did not receive FLU_5CMC.

counteracted by prostaglandin E_2 or H_2O_2. Presumably, MØ derived nonspecific immunoregulatory molecules functioned only at close range, or only on B cells which had also received an Ig receptor and/or Fc receptor mediated signal, since tolerance was hapten specific and Fc dependent. The phenomenon described by Goldings may only be applicable to Fc dependent tolerance induction. We could demonstrate no role for IL-1 or prostaglandins in A cell dependent, CMC/MC induced tolerance, despite the importance of such molecules in the activation (*Chapter V*) and nonspecific suppression (Thompson *et al*, 1984b; Jelinek *et al*, 1985) of B cells.

The phenomenon we have reported here is most likely dissimilar to the recently reported role of antigen presenting MØ in the induction of tolerance to SGG (Phipps and Scott, 1983; Phipps *et al*, 1984). In the latter system, the MØ most likely served to present the tolerogen in a multivalent, more effectively tolerogenic fashion. This effect was Fc dependent, since non-gamma globulin antigens were not tolerogenic (Phipps and Scott, 1983) and only FcR^+ A cells presented FLU-SGG in a tolerogenic fashion (Phipps *et al*, 1984). The FcR^-, Ia^+ dendritic cell-like line P388AD.2 and certain Ia^+ FcR^+ MØ-like lines presented FLU-SGG in an immunogenic fashion. In contrast to the work of Phipps and Scott (1983), we could demonstrate no B cell tolerance in the absence of A cells (Figure 7), even when several-fold higher concentrations of TMP-MC were used (Table 11). This precludes the possibility that hapten specific B cells recognize antigen presented in a nonimmunogenic or tolerogenic manner by other B cells. Thus, it is unlikely that A cells are merely serving to present tolerogen in a concentrated fashion in our system. The inability of glutaraldehyde fixed A cells from TNP-CMC tolerant mice to render B cells hapten specifically unresponsive further suggests that A cells actively deliver a negative signal to certain B cells. This most likely occurs *via* direct A cell-B cell interaction, since hapten specifically suppressive molecules could not be detected in the supernatants of TNP-MC treated A cells (Figure 22).

We postulate that the hapten carriers CMC and MC bind to the A cell surface membrane, perhaps by receptors analogous to previously described mannose receptors (Stahl *et al*, 1978), thereby rendering this cell specifically suppressive only for B cells recognizing the carrier bound hapten. Since A cell dependent TI B cell activation does not require direct A cell-B cell contact (*Chapter V*), only B cells focussed onto A cells by hapten-CMC/MC will be tolerized. Experiments in which B cells are placed in close proximity to unhaptenated MC treated A cells by gentle centrifugation underscore the importance of hapten focussing in this system, since such experimental manipulation did not result in the generalized loss of B cell immunocompetence (data not shown).

There is abundant evidence that distinct MØ subpopulations may under certain conditions exert inhibitory influences on various cell types. Indeed, evidence suggests that MØ mediated cytostatic and cytotoxic mechanisms involve the synergistic interaction between proteases and H_2O_2 which are generated by activated MØ and transferred to the target cells (reviewed in Adams and Nathan, 1983; Adams and Hamilton, 1984). MØ are known to generate superoxides under appropriate stimulatory conditions, such as in the presence of certain bacteria and organic compounds (Adams and Hamilton, 1984). CMC and MC, which, like some bacterial cell wall components, have polymeric carbohydrate structures, may thus have the ability to stimulate certain MØ to produce superoxide or peroxide-like substances. Following a protocol used by Aune and Pierce (1981c) for the purposes of interfering with oxidative processes, we were able to reverse "tolerance" in A cells or spleen cells by treating them with the reducing agent KI (Tables 14 and 15). This is consistent with reports that iodoacetate used at similar concentrations inhibits the respiratory burst necessary for the generation of reactive oxygen intermediates (Sbarra and Karnovsky, 1959; Iyer *et al*, 1961; Takanaka and O'Brien, 1975). However, pretreatment with KI of TNP-MC *in vitro* did not reduce the specific tolerogenic properties of this compound (data not shown). Thus, A cell oxidative processes may be responsible for the clonal deletion of hapten specific B cells interacting with surface bound hapten-CMC/MC. Consistent with this, spleen cells from FLU tolerant mice contained barely detectable levels of FLU binding mIg^+ cells (Figures 24 and 26; Table 18); B cells have been shown to be extremely susceptible to H_2O_2 mediated cytotoxicity (Farber *et al*, 1984). Further investigation is required to elucidate the molecular basis of negative signalling in our system.

In summary, these results suggest that hapten specific CMC/MC induced tolerance is mediated by a novel, A cell dependent mechanism, possibly involving the clonal deletion of B cells. Tolerance could not be explained within the framework of existing theories of B cell tolerance. For example, since CMC/MC are not polyclonal B cell activators (von Borstel *et al*, 1983) the one nonspecific model (Coutinho and Möller, 1974) cannot be relevant. In disagreement with a major prediction of the two signal model (Bretscher and Cohn, 1970), neither IL-1 (Table 12, line 7), nor carrier specific T cell help (Table 17) interfered with the induction of tolerance by hapten-CMC/MC conjugates. Although the deficiency of FLU binding B cells in FLU-CMC tolerant mice would seem to be consistent with a deletional mechanism for CMC/MC induced tolerance, clonal deletion theory places no constraints upon the molecular nature of the antigen or its mode of presentation. The abrogation of tolerogenic capactiy by oxidation-reduction of the CMC/MC carrier (Diener *et al*, 1981; von Borstel *et al*, 1983) and the A cell dependence of CMC/MC induced tolerance are therefore not consistent with classical clonal deletion/abortion theory.

The relevance of A cell dependent clonal deletion as a mechanism of self tolerance awaits confirmation of its importance in experimental tolerance to other antigens. However, in view of our previous work demonstrating B cell unresponsiveness to CMC conjugates of penicllin derivatives, the potential clinical applications of such potent, yet completely nonimmunogenic tolerogens are immediately apparent. A cells conditioned *in vitro* to induce specific unresponsiveness may be particularly useful in this regard.

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